DESCRIPTION

# METHOD OF AMPLIFYING NUCLEIC ACID

## Technical Field

[0001]

The present invention relates to a method for detecting a target nucleic acid which is useful in a field of clinical medicine, and a method for synthesizing a DNA which is useful in a field of genetic engineering. The present invention relates to a method for amplifying a nucleic acid as a template and a method for detecting a target nucleic acid amplified according said method.

## Background Art

[0002]

DNA synthesis is used for various purposes in studies in a field of genetic engineering. Most of the DNA syntheses with the exception of those of short-chain DNAs (e.g., oligonucleotides) are carried out using an enzymatic method in which a DNA polymerase is utilized.

For example, according to the polymerase chain reaction (PCR) method, it is required to repeat reactions at a high temperature and a low temperature several times in order to regenerate single-stranded target molecules for

the subsequent amplification cycles. Since the reactions are restricted by temperatures as described above, the reaction system needs to be conducted using discontinuous phases or cycles.

[0003]

Accordingly, this method requires the use of an expensive thermal cycler that can strictly adjust a wide range of temperatures over time. Furthermore, the reaction requires time for adjusting the temperature to the two or three predetermined ones. The loss of time increases in proportion to the cycle number.

[0004]

Then, nucleic acid amplification methods that can be carried out under isothermal conditions have been developed in order to solve the above-mentioned problems.

Examples of the methods that can be carried out under isothermal conditions include the Strand Displacement Amplification (SDA) method (see, for example, Patent Document 1), the Rolling Circle Amplification (RCA) method (see, for example, Patent Document 2), the Loop-mediated isothermal AMPlification (LAMP) method (see, for example, Patent Document 3), the Isothermal and Chimeric primer-initiated Amplification of Nucleic acids (ICAN) method (see, for example, Patent Document 4) and the various modified SDA methods (see, for example, Patent Documents 5-8).

[0005]

In a reaction of such an isothermal method of nucleic acid amplification or oligonucleotide synthesis, extension from a primer and annealing of a primer to a single-stranded extension product (or to an original target sequence) followed by extension from the primer take place in parallel in a reaction mixture incubated at a constant temperature.

[0006]

The SDA method as described in Patent Document 1 is a method for amplifying a target nucleic acid sequence (and a complementary strand thereof) in a sample by displacement of double strands with the aid of a DNA polymerase and a restriction endonuclease. This method requires four primers used for amplification, and two of them need to be constructed to contain a recognition site for the restriction endonuclease. The method requires the use of a modified deoxyribonucleotide triphosphate as a substrate for DNA synthesis in large quantities. The modified deoxyribonucleotide triphosphate is exemplified by (α-S) deoxyribonucleotide triphosphate in which an oxygen atom in a phosphate group at α-position is replaced by a sulfur atom (S).

[0007]

The RCA method as described in Patent Document 2

is a nucleic acid amplification method in which a circular DNA is used as a template. The resulting amplification product has a sequence in which amplified regions are repeated several times, and serves as a new template. Consequently, the final amplification product has a branched structure called "branching". Regarding the RCA method, it has been reported that suppression of generation of a ladder-like amplification product in a side reaction is important for increasing the amplification efficiency (see, for example, Non-patent Document 1).

[0008]

The LAMP method as described in Patent Document 3 requires four primers for amplification. The resulting amplification products are ladder-like DNAs with varying sizes in which target regions to be amplified are repeated. In the DNA, the target regions are connected to each other in alternate directions.

[0009]

The ICAN method as described in Patent Document 4 is a nucleic acid amplification method in which a chimeric oligonucleotide primer is used.

[0010]

The modified SDA method as described in Patent Document 5 is a DNA amplification method in which a chimeric oligonucleotide primer is used. The chimeric

oligonucleotide primer is composed of RNA and DNA and has, as an essential element, a structure in which DNA is positioned at least at the 3' terminus.

[0011]

The modified SDA method as described in Patent Document 6 requires the use of a restriction enzyme that generates a 3'-protruding terminus.

[0012]

The modified SDA method as described in Patent Document 7 requires the use of at least two pairs of primers.

[0013]

The modified SDA method as described in Patent Document 8 requires the use of at least two pairs of primers and at least one modified deoxyribonucleotide triphosphate.

[0014]

Isothermal nucleic acid amplification methods have been developed as described above, although these methods also have problems concerning the amplification efficiencies, the detection sensitivities and the like. Then, improved isothermal nucleic acid amplification methods have been developed in order to solve the problems (see, for example, Patent Documents 9-11).

[0015]

The method as described in Patent Document 9 is a nucleic acid amplification method using the following: a primer that has a promoter for an RNA polymerase or a part thereof attached at the 5' terminus of a reverse transcription primer or a primer for nucleic acid amplification, and further has an arbitrary oligonucleotide attached at the 5' terminus which contains at least five nucleotides; and a third primer that anneals to an upstream region or a portion of the promoter for an RNA polymerase in the primer.

[0016]

The method as described in Patent Document 10 is a nucleic acid amplification method in which at least two complementary primers or at least two primer pairs are used.

This method is a method in which the amount of generated amplification product is increased by the use of plural primers or plural primer pairs. It is necessary to use more primers or primer pairs for increasing the productivity.

[0017]

The method as described in Patent Document 11 is a method according to the method as described in Patent Document 12 using the following in combination: a chimeric oligonucleotide primer; and an upstream oligonucleotide primer which hybridizes to a portion in a template nucleic

acid 3' to the position at which the primer hybridizes.

According to this method, a ladder-forming oligonucleotide primer consists only of a sequence that hybridizes to a template strand.

[0018]

The conventional isothermal nucleic acid amplification methods as described above still have various problems. Thus, an efficient and highly sensitive method has been desired.

[0019]

Patent Document 1: JP-B 7-114718

Patent Document 2: WO 97/19193

Patent Document 3: WO 00/28082

Patent Document 4: WO 00/56877

Patent Document 5: US 5,824,517

Patent Document 6: WO 99/09211

Patent Document 7: WO 95/25180

Patent Document 8: WO 99/49081

Patent Document 9: WO 95/03426

Patent Document 10: WO 95/25180

Patent Document 11: JP-A 2003-52380

Non-patent Document 1: Hafner, G.J. et al., BioTechniques, vol.30 (2001) p.852-867

## Disclosure of Invention

Problems to be Solved by the Invention

[0020]

The main object of the present invention is to provide a method for amplifying a target nucleic acid by which a target nucleic acid in a sample is specifically amplified with high sensitivity, as well as a composition and a kit for this method.

Means to Solve the Problems

[0021]

The present inventors have studied intensively in order to solve the above-mentioned problems. As a result, the present inventors have found that a ladder-like product is generated in an ICAN reaction if substantially identical sequences are present in a nucleic acid as a template, and a primer is designed for a sequence within a region between the sequences. In the ladder-like product, target regions are polymerized through the identical sequences in one direction. The present inventors have further found that the sensitivity, the amplification efficiency and the reaction velocity of an ICAN reaction can be increased by positively converting the amplification products into ladder-like ones. The conversion is accomplished by using a chimeric oligonucleotide primer and a ladder-forming oligonucleotide primer which contains a specific nucleotide

sequence. Thus, the present invention has been completed.

[0022]

The first aspect of the present invention relates to a method for amplifying a nucleic acid, the method comprising the steps of:

(A) preparing a reaction mixture selected from:

(a) a nucleic acid as a template, a deoxyribonucleotide triphosphate, a DNA polymerase having a strand displacement activity, at least two chimeric oligonucleotide primers, at least one ladder-forming oligonucleotide primer and an RNase H; or

(b) a nucleic acid as a template, a deoxyribonucleotide triphosphate, a DNA polymerase having a strand displacement activity, at least two chimeric oligonucleotide primers and an RNase H, wherein one of the chimeric oligonucleotide primers serves as a ladder-forming oligonucleotide primer,

wherein each chimeric oligonucleotide primer contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, and the ribonucleotide is positioned at the 3' terminus or on the 3'-terminal side of the primer,

wherein the chimeric oligonucleotide primers comprise at least a first chimeric oligonucleotide primer which is complementary to a nucleotide sequence of the

nucleic acid as a template and a second chimeric oligonucleotide primer which is homologous to a nucleotide sequence of the nucleic acid as a template, and

wherein the ladder-forming oligonucleotide primer has a sequence complementary to a region of the nucleic acid as a template that is complementary to the first chimeric oligonucleotide primer and/or a nucleotide sequence 3' to said region, and has, on its 5' side, a sequence complementary to: a nucleotide sequence on the 5' side of the second chimeric oligonucleotide primer which is homologous to the nucleic acid as a template; a nucleotide sequence of the nucleic acid as a template corresponding to a region 5' to the 5' terminus of the portion homologous to the second chimeric oligonucleotide primer; or both; and

(B) incubating the reaction mixture for a sufficient time to generate a ladder-like amplification product under constant-temperature conditions under which specific annealing of the primer to the nucleic acid as a template, a reaction of synthesizing an extended strand and a strand displacement reaction by the DNA polymerase, as well as a reaction of cleaving an extended strand by the RNase H take place.

[0023]

According to the first aspect, the nucleic acid as a template may be an RNA, and the nucleic acid may be

treated beforehand with a deoxyribonucleotide triphosphate, a DNA polymerase having a reverse transcription activity and at least one ladder-forming oligonucleotide primer to convert the nucleic acid into a reverse transcription product.

[0024]

According to the first aspect, the reaction mixture in step (A) may further contain a DNA polymerase having a reverse transcription activity.

[0025]

According to the first aspect, the nucleic acid as a template may be an mRNA.

[0026]

According to the first aspect, a single DNA polymerase having a reverse transcription activity and a strand displacement activity may be used.

[0027]

The second aspect of the present invention relates to a composition for the method of the first aspect, which contains at least one chimeric oligonucleotide primer and/or at least one ladder-forming oligonucleotide primer.

[0028]

The third aspect of the present invention relates to a kit for the first aspect, which contains at least one chimeric oligonucleotide primer and/or at least one ladder-

forming oligonucleotide primer.

[0029]

The fourth aspect of the present invention relates to a method for detecting a target nucleic acid, the method comprising the steps of:

(a) amplifying a target nucleic acid according to the method of the first aspect; and

(b) detecting the target nucleic acid amplified in the above step.

[0030]

The fifth aspect of the present invention relates to an oligonucleotide primer used for the method of the first aspect, which has, on its 5' side, a sequence complementary to: a nucleotide sequence on the 5' side of a primer that is homologous to a nucleic acid as a template; a nucleotide sequence of the nucleic acid as a template corresponding to a region 5' to the 5' terminus of a portion homologous to the second chimeric oligonucleotide primer; or both.

Effects of the Invention

[0031]

The present invention provides an amplification method superior to conventional isothermal nucleic acid amplification methods as well as a composition and a kit

containing a primer used for the method.

## Brief Description of Drawings

[0032]

Figure 1 illustrates positional relationships among the first chimeric oligonucleotide primer, the second oligonucleotide primer and the ladder-forming oligonucleotide primers used in Example 2-(1)-(A). (a) represents the first chimeric oligonucleotide primer, (b) to (d) represent the ladder-forming oligonucleotide primers, and (e) represents the second chimeric oligonucleotide primer. Each of the parts 1 in (b) to (d) has a sequence homologous to the part 1 in the complementary strand of a portion 5' upstream of (e).

Figure 2 illustrates amplification curves for varying copy numbers. The horizontal axis in Figure 2 represents the reaction cycle number. In Figure 2, a, b, c, d, e and f represent amplification curves obtained using $10^5$ copies, $10^4$ copies, $10^3$ copies, $10^2$ copies, $10^1$ copies and $10^0$ copy of the template, respectively.

## Best Mode for Carrying Out the Invention

[0033]

As used herein, a deoxyribonucleotide (also referred to as a dN) refers to a nucleotide of which the

sugar portion is composed of D-2-deoxyribose. Examples thereof include ones having adenine, cytosine, guanine or thymine as the base portion. Furthermore, the deoxyribonucleotides also include a deoxyribonucleotide having a modified base such as 7-deazaguanosine, a deoxyribonucleotide having a functional group that can be used for immobilization and a deoxyribonucleotide analog such as deoxyinosine nucleotide.

[0034]

As used herein, a ribonucleotide (also referred to as an N) refers to a nucleotide of which the sugar portion is composed of D-ribose. The ribonucleotides include ones having adenine, cytosine, guanine or uracil as the base portion. The ribonucleotides also include modified ribonucleotides such as a modified ribonucleotide in which an oxygen atom in a phosphate group at α-position is replaced by a sulfur atom (S) (also referred to as an (α-S) ribonucleotide or an (α-S) N) or other derivatives.

[0035]

As used herein, a chimeric oligonucleotide primer refers to an oligonucleotide primer that has a ribonucleotide positioned at the 3' terminus or on the 3'-terminal side of the primer, can be used to extend a nucleic acid strand according to the method of the present invention, can be cleaved with an RNase H, and can be used

to effect a strand displacement reaction. The chimeric oligonucleotide primers according to the present invention include any one having the above-mentioned constitution.

A chimeric oligonucleotide primer used according to the present invention contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog. The primers include oligoribonucleotide primers containing an unmodified ribonucleotide and/or a modified ribonucleotide.

A chimeric oligonucleotide primer used in the method of the present invention has a nucleotide sequence substantially complementary to a part of the nucleotide sequence of a nucleic acid as a template. As used herein, "a substantially complementary nucleotide sequence" means a nucleotide sequence that is capable of annealing to a DNA as a template under reaction conditions used.

[0036]

As used herein, 3'-terminal side refers to a portion from the center to the 3' terminus of a nucleic acid (e.g., a primer). Likewise, 5'-terminal side refers to a portion from the center to the 5' terminus of a nucleic acid.

[0037]

As used herein, an upstream region refers to a region of a template strand that is 5' to the 5' terminus

of a nucleotide sequence of a chimeric oligonucleotide primer, wherein the template strand is homologous to the chimeric oligonucleotide primer to be used. Thus, it corresponds to a region of the strand complementary to the template strand that is 3' to the 3' terminus of the sequence complementary to the nucleotide sequence of the chimeric oligonucleotide primer.

[0038]

As used herein, a nucleotide sequence for ladder formation refers to a nucleotide sequence complementary to: a nucleotide sequence on the 5' side of a chimeric oligonucleotide primer used in the method of the present invention; a nucleotide sequence of a nucleic acid as a template corresponding to a region upstream of (i.e., 5' to) the 5' terminus of a portion homologous to the chimeric oligonucleotide primer; or both.

[0039]

As used herein, a ladder-forming oligonucleotide primer refers to one containing the above-mentioned nucleotide sequence for ladder formation on the 5' side of the primer.

[0040]

Positions in a nucleotides sequence are indicated herein as follows: the 5' terminus of a chimeric oligonucleotide primer used in the method of the present

invention or a position corresponding to the position in the template strand to which the chimeric oligonucleotide primer anneals is defined as "0". The positions towards the 3' terminus of the chimeric oligonucleotide primer are represented by integers "+1", "+2", ... The position 1 nucleotide upstream of the position "0" in the nucleic acid as a template is defined as "-1". The positions towards upstream are represented by integers "-2", "-3", ...

[0041]

As used herein, an RNase H (ribonuclease H) may be any one that acts on a double-stranded DNA generated by DNA extension from a chimeric oligonucleotide primer that has been annealed to a nucleic acid as a template, and specifically cleaves it at a ribonucleotide portion in the primer.

[0042]

As used herein, a DNA polymerase refers to an enzyme that synthesizes a new DNA strand using another DNA strand as a template (a DNA-dependent DNA polymerase) or an enzyme that synthesizes a DNA strand complementary to an RNA strand using the RNA strand as a template (an RNA-dependent DNA polymerase). The DNA polymerases include naturally occurring DNA polymerases and variant enzymes each having the above-mentioned activity. For example, such enzymes include a DNA polymerase having a strand

displacement activity, a DNA polymerase lacking a 5'→3' exonuclease activity and a DNA polymerase additionally having a reverse transcriptase activity or an RNase H activity.

[0043]

As used herein, "a strand displacement activity" refers to an activity that is capable of effecting a strand displacement, that is, that can proceed with DNA duplication on the basis of a sequence of a nucleic acid as a template while displacing a DNA strand to release the complementary strand that has been annealed to the template strand. In addition, the DNA strand released from the nucleic acid as a template as a result of strand displacement is referred to as "a displaced strand" herein.

[0044]

As used herein, "a reverse transcription activity" refers to an activity that is capable of synthesizing a DNA strand complementary to an RNA strand using the RNA strand as a template.

[0045]

(1) The method for amplifying a target nucleic acid of the present invention

It is possible to carry out the method of the present invention using at least one chimeric oligonucleotide primer and at least one ladder-forming

oligonucleotide primer in combination with an RNase H and a DNA polymerase. Alternatively, it is possible to use a DNA polymerase having an RNase H activity under conditions under which its RNase H activity is exhibited.

[0046]

A nucleic acid is successively amplified under isothermal conditions according to the method of the present invention. "Successively" means that a reaction proceeds without a change in the reaction temperature or the composition of the reaction mixture. As used herein, "isothermal" means conditions of a substantially constant temperature under which an enzyme and a nucleic acid strand function in each step as described below.

[0047]

In one embodiment of the method for amplifying a target nucleic acid of the present invention, an exemplary method for amplifying a nucleic acid comprises the steps of:

(a) treating a nucleic acid as a template with a deoxyribonucleotide triphosphate, a DNA polymerase having a reverse transcription activity and at least one ladder-forming oligonucleotide primer to obtain a reverse transcription product;

(b) preparing a reaction mixture by mixing the reverse transcription product, a DNA polymerase having a

strand displacement activity, at least two chimeric oligonucleotide primers and an RNase H; and

(c) incubating the reaction mixture for a sufficient time to generate a ladder-like amplification product under constant-temperature conditions under which a reaction of synthesizing an extended strand and a strand displacement reaction by the DNA polymerase, as well as a reaction of cleaving an extended strand by the RNase H take place.

Each chimeric oligonucleotide primer contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, and the ribonucleotide is positioned at the 3' terminus or on the 3'-terminal side of the primer.

The chimeric oligonucleotide primers comprise at least a first chimeric oligonucleotide primer which is complementary to a nucleotide sequence of the nucleic acid as a template and a second chimeric oligonucleotide primer which is homologous to a nucleotide sequence of the nucleic acid as a template.

The ladder-forming oligonucleotide primer is complementary to a region of the nucleic acid as a template that is complementary to the first chimeric oligonucleotide primer and a nucleotide sequence 3' to said region, and has, on its 5' side, a nucleotide sequence complementary to: a

nucleotide sequence on the 5' side of the second chimeric oligonucleotide primer which is homologous to the nucleic acid as a template; a nucleotide sequence of the nucleic acid as a template corresponding to a region upstream of (i.e., 5' to) the 5' terminus of the portion homologous to the second chimeric oligonucleotide primer; or both.

[0048]

In another embodiment of the present invention, an exemplary method for amplifying a nucleic acid comprises the steps of:

(a) preparing a reaction mixture by mixing a nucleic acid as a template, a deoxyribonucleotide triphosphate, a DNA polymerase having a reverse transcriptase activity, a DNA polymerase having a strand displacement activity, at least two chimeric oligonucleotide primers, at least one ladder-forming oligonucleotide primer and an RNase H; and

(b) incubating the reaction mixture for a sufficient time to generate a ladder-like amplification product under constant-temperature conditions under which specific annealing of the primer to the nucleic acid as a template, a reaction of synthesizing an extended strand and a strand displacement reaction by the DNA polymerase, as well as a reaction of cleaving an extended strand by the RNase H take place.

Each chimeric oligonucleotide primer contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, and the ribonucleotide is positioned at the 3' terminus or on the 3'-terminal side of the primer.

The chimeric oligonucleotide primers comprise at least a first chimeric oligonucleotide primer which is complementary to a nucleotide sequence of the nucleic acid as a template and a second chimeric oligonucleotide primer which is homologous to a nucleotide sequence of the nucleic acid as a template.

The ladder-forming oligonucleotide primer is complementary to a region of the nucleic acid as a template that is complementary to the first chimeric oligonucleotide primer and a nucleotide sequence 3' to said region, and has, on its 5' side, a nucleotide sequence complementary to: a nucleotide sequence on the 5' side of the second chimeric oligonucleotide primer which is homologous to the nucleic acid as a template; a nucleotide sequence of the nucleic acid as a template corresponding to a region upstream of (i.e., 5' to) the 5' terminus of the portion homologous to the second chimeric oligonucleotide primer; or both.

[0049]

In a further embodiment of the present invention, an exemplary method for amplifying a nucleic acid comprises

the steps of:

(a) preparing a reaction mixture by mixing a nucleic acid as a template, a deoxyribonucleotide triphosphate, a DNA polymerase having a strand displacement activity, at least two chimeric oligonucleotide primers, at least one ladder-forming oligonucleotide primer and an RNase H; and

(b) incubating the reaction mixture for a sufficient time to generate a ladder-like amplification product under constant-temperature conditions under which specific annealing of the primer to the nucleic acid as a template, a reaction of synthesizing an extended strand and a strand displacement reaction by the DNA polymerase, as well as a reaction of cleaving an extended strand by the RNase H take place.

Each chimeric oligonucleotide primer contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, and the ribonucleotide is positioned at the 3' terminus or on the 3'-terminal side of the primer.

The chimeric oligonucleotide primers comprise at least a first chimeric oligonucleotide primer which is complementary to a nucleotide sequence of the nucleic acid as a template and a second chimeric oligonucleotide primer which is homologous to a nucleotide sequence of the nucleic

acid as a template.

The ladder-forming oligonucleotide primer is complementary to a region of the nucleic acid as a template that is complementary to the first chimeric oligonucleotide primer and a nucleotide sequence 3' to said region, and has, on its 5' side, a nucleotide sequence complementary to: a nucleotide sequence on the 5' side of the second chimeric oligonucleotide primer which is homologous to the nucleic acid as a template; a nucleotide sequence of the nucleic acid as a template corresponding to a region upstream of (i.e., 5' to) the 5' terminus of the portion homologous to the second chimeric oligonucleotide primer; or both.

[0050]

In the above-mentioned embodiments, if the nucleic acid as a template is a single-stranded one, the first chimeric oligonucleotide primer has a nucleotide sequence complementary to the strand; the second chimeric oligonucleotide primer has a nucleotide sequence complementary to a strand complementary to the strand, that is, a nucleotide sequence homologous to the strand; and the ladder-forming chimeric oligonucleotide primer has a sequence nucleotide complementary to the strand and, on its 5' side, a nucleotide sequence complementary to: a nucleotide sequence on the 5' side of the second chimeric oligonucleotide primer which is homologous to the strand; a

nucleotide sequence corresponding to a region upstream of the 5' terminus of the second chimeric oligonucleotide primer; or both. It is needless to say that, also in case where the nucleic acid as a template is a double-stranded one, the primers can be designed for one of the strands in a similar manner.

[0051]

In any one of the above-mentioned embodiments, the ladder-forming oligonucleotide primer may be replaced by a chimeric oligonucleotide primer. In this case, the chimeric oligonucleotide primer can supplement the function of the ladder-forming oligonucleotide primer. Thus, an oligonucleotide primer and/or a chimeric oligonucleotide primer may be preferably used as long as it can contribute to ladder formation.

Although it is not intended to limit the present invention, for example, a first chimeric oligonucleotide primer can be used as a ladder-forming oligonucleotide primer. In this case, the first chimeric oligonucleotide primer has, on its 5' side, a nucleotide sequence complementary to: a nucleotide sequence on the 5' side of a second chimeric oligonucleotide primer which is homologous to a nucleic acid as a template; a nucleotide sequence corresponding to a region upstream of the 5' terminus of the second chimeric oligonucleotide primer; or both.

[0052]

dNTPs (a mixture of dATP, dCTP, dGTP and dTTP) which are used for the PCR method or the like can be preferably used as nucleotide triphosphates that serve as substrates in the extension reaction in the method. In addition, dUTP may be used as a substrate. The dNTPs may contain an analog of dNTP (deoxyribonucleotide triphosphate) such as 7-deaza-dGTP, or a nucleotide triphosphate such as dITP as long as it can serve as a substrate for the DNA polymerase to be used. A derivative of a dNTP or a dNTP analog may be used. A derivative having a functional group such as a dUTP having an amino group may be contained. A chimeric oligonucleotide primer is used in the method. The primer can be prepared, for example, using a DNA synthesizer according to a conventional synthesis method.

[0053]

The nucleic acid (DNA or RNA) used as a template in the method of the present invention may be prepared or isolated from any sample that may contain the nucleic acid. Alternatively, the sample may be used directly in the nucleic acid amplification reaction of the present invention. Examples of the samples that may contain the nucleic acid include, but are not limited to, samples from organisms such as a whole blood, a serum, a buffy coat, a

urine, feces, a cerebrospinal fluid, a seminal fluid, a saliva, a tissue (e.g., a cancerous tissue or a lymph node) and a cell culture (e.g., a mammalian cell culture or a bacterial cell culture), samples that contain a nucleic acid such as a viroid, a virus, a bacterium, a fungi, a yeast, a plant and an animal, samples suspected to be contaminated or infected with a microorganism such as a virus or a bacterium (e.g., a food or a biological formulation), and samples that may contain an organism such as a soil and a waste water. The sample may be a nucleic acid-containing preparation obtained by processing the above-mentioned sample according to a known method. For example, a cell destruction product or a sample obtained by fractionating the product, a nucleic acid in the sample, or a sample in which a specific nucleic acid molecule population (e.g., mRNA) is enriched can be used as such a preparation according to the present invention. Also, a nucleic acid such as a DNA or an RNA obtained by amplifying a nucleic acid contained in the sample according to a known method can be preferably used.

[0054]

A nucleic acid-containing preparation can be prepared from the above-mentioned material by using, for example, lysis with a detergent, sonication, shaking/stirring using glass beads or a French press,

although it is not intended to limit the present invention. In some cases, it is advantageous to further process the preparation to purify the nucleic acid (e.g., in case where an endogenous nuclease exists). In such cases, the nucleic acid is purified using a known means such as phenol extraction, chromatography, ion exchange, gel electrophoresis or density-gradient centrifugation.

[0055]

If it is desired to amplify a nucleic acid having a sequence derived from an RNA, the method of the present invention may be conducted using, as a template, a cDNA synthesized by a reverse transcription reaction in which the RNA is used as a template. Any RNA for which one can make a primer to be used in a reverse transcription reaction can be applied to the method of the present invention, including total RNA in a sample, an RNA molecule population such as mRNA, tRNA or rRNA, and specific RNA molecular species.

[0056]

Any enzyme that has an activity of synthesizing a cDNA using an RNA as a template can be used in the reverse transcription reaction without limitation. Examples thereof include reverse transcriptases originating from various sources such as avian myeloblastosis virus-derived reverse transcriptase (AMV RTase), Molony murine leukemia

virus-derived reverse transcriptase (M-MLV RTase) and Rous-associated virus 2 reverse transcriptase (RAV-2 RTase). In addition, a DNA polymerase additionally having a reverse transcription activity can be used. An enzyme having a reverse transcription activity at a high temperature is preferable for the purpose of the present invention. For example, a DNA polymerase from a bacterium of the genus *Thermus* (e.g., Tth DNA polymerase), a DNA polymerase from a thermophilic bacterium of the genus *Bacillus* or the like can be used. Although it is not intended to limit the present invention, for example, DNA polymerases from thermophilic bacteria of the genus *Bacillus* such as a DNA polymerase from B. st (Bst DNA polymerase) and Bca DNA polymerase are preferable. For example, Bca DNA polymerase does not require a manganese ion for a reverse transcription reaction. Furthermore, it can synthesize a cDNA while suppressing secondary structure formation of a template RNA under high-temperature conditions. Both a naturally occurring one and a variant of the enzyme having a reverse transcriptase activity can be used as long as they have the activity.

[0057]

Although it is not intended to limit the present invention, an oligonucleotide of preferably about 6 nucleotides to about 50 nucleotides, more preferably about

10 nucleotides to about 40 nucleotides, still more preferably about 12 nucleotides to about 30 nucleotides can be used as a chimeric oligonucleotide primer according to the method of the present invention. It is preferable that the nucleotide sequence of the chimeric oligonucleotide primer is complementary to a template nucleic acid such that it anneals to the nucleic acid as a template under reaction conditions to be used. The primer contains a sequence recognized by an RNase H, which is used in a step as described below, at the 3' terminus or on the 3'-terminal side.

[0058]

Although it is not intended to limit the present invention, for example, an oligonucleotide having a structure represented by the following general formula can be used as a primer in a DNA synthesis method according to the present invention:

General formula: 5'-dNa-Nb-dNc-3'

(in the general formula, "dN" represents deoxyribonucleotide and/or nucleotide analog, and "N" represents unmodified ribonucleotide and/or modified ribonucleotide, wherein an analog of a nucleotide or a derivative of a nucleotide (a modified nucleotide) may be contained for each nucleotide as long as the function is not impaired, and some of dNs in "dNa" may be replaced by

Ns).

In the above-mentioned general formula, for example, "a" is an integer of 5 or more, preferably 6 or more, more preferably 8 or more; "b" is an integer of 1 or more, for example 1-15, preferably 1-10, more preferably 1-7, still more preferably 1-5; and "c" may be 0 or c may be an integer of 1 or more, preferably 0-5, more preferably 0-3, although it is not intended to limit the present invention.

[0059]

According to the method of the present invention, a ladder-forming oligonucleotide primer may be used for obtaining a ladder-like amplification product. At least one selected from the group consisting of a deoxyribonucleotide, a ribonucleotides and a nucleotide analog can be preferably used for the primer.

[0060]

It is preferable to use, as a ladder-forming oligonucleotide primer used in the method of the present invention, one having, on its 5' side, an attached nucleotide sequence complementary to: a nucleotide sequence on the 5' side of a second chimeric oligonucleotide primer which is homologous to a nucleic acid as a template; a nucleotide sequence corresponding to a region upstream of the 5' terminus of the second chimeric oligonucleotide

primer; or both.

[0061]

If an RNA is used as a template according to the present invention, a ladder-forming oligonucleotide primer may be used as a primer for reverse transcription. There is no specific limitation as long as it anneals to the template RNA under reaction conditions to be used. The primer may be a primer having a nucleotide sequence that is complementary to a specific template RNA (a specific primer), an oligo-dT (deoxythymine) primer and a primer having a random sequence (a random primer). In view of specific annealing, the length of the portion of a sequence complementary to a part of the nucleotide sequence of the template nucleic acid in the ladder-forming oligonucleotide primer is preferably 6 nucleotides or more, more preferably 9 nucleotides or more. In view of oligonucleotide synthesis, the length is preferably 100 nucleotides or less, more preferably 30 nucleotides or less. There is no specific limitation as long as it can be used for a reverse transcription reaction from an RNA.

[0062]

There is no specific limitation concerning the ladder-forming oligonucleotide primer as long as it can contribute to extension of a DNA strand in a ladder-like pattern under conditions to be used. The primer preferably

has a nucleotide sequence complementary to: a nucleotide sequence on the 5' side of a chimeric oligonucleotide primer that is homologous to a nucleic acid as a template; a nucleotide sequence corresponding to a region upstream of the 5' terminus of the chimeric oligonucleotide primer; or both. Although it is not intended to limit the present invention, the length of the sequence complementary to the region upstream of the chimeric oligonucleotide primer is preferably about 3 to about 100 nucleotide, more preferably about 4 to about 50 nucleotides, still more preferably about 5 to about 20 nucleotides.

[0063]

Although it is not intended to limit the present invention, the sequence complementary to a region upstream of the chimeric oligonucleotide primer is a sequence of 3 nucleotides or more selected from position +20 to position -100, preferably position +15 to position -80, more preferably position +10 to position -60, still more preferably position +5 to position -40. Examples of such nucleotide sequences include, but are not limited to, regions starting from position -1, position -11 or position -21.

[0064]

There is no specific limitation concerning the portion to which the ladder-forming oligonucleotide primer

anneals as long as the function can be exerted. The portion may be a portion overlapping entirely or by one or more nucleotide(s) with, adjacent to, or at a distance of 1 to 40 nucleotide(s) from the chimeric oligonucleotide primer.

[0065]

Although it is not intended to limit the present invention, the GC content of the ladder-forming oligonucleotide primer is preferably 40% or more.

[0066]

Furthermore, the ladder-forming oligonucleotide primer used in the method of the present invention may contain a nucleotide analog or other substances. That is, one or more nucleotide analog(s) can be contained in the ladder-forming oligonucleotide primer of the present invention as long as the function of the primer for effecting a polymerase extension reaction from the 3' terminus by the action of a DNA polymerase is not abolished. Plural types of the nucleotide analogs can be used in combination. Examples of the nucleotide analogs that can be used include, but are not limited to, deoxyinosine nucleotide, deoxyuracil nucleotide, a nucleotide analog having a modified base such as 7-deazaguanine, a nucleotide analog having a ribose derivative and the like. Furthermore, the chimeric oligonucleotide primer used

according to the present invention may contain a deoxynucleotide, a ribonucleotide or a nucleotide analog having one of various modifications such as addition of a labeled compound or addition of a functional group for immobilization as long as the functions as described above are retained.

Incorporation of a nucleotide analog into a primer is effective for suppressing formation of a high-order structure of the primer itself and stabilizing a form annealed to a template.

[0067]

The chimeric oligonucleotide primer and the ladder-forming oligonucleotide primer used in the method of the present invention can be synthesized to have arbitrary nucleotide sequences using, for example, Applied Biosystems Inc. (ABI) 394 DNA synthesizer according to the phosphoramidite method. Alternatively, any methods including the phosphate triester method, the H-phosphonate method and the thiophosphonate method may be used to for the synthesis.

[0068]

In another embodiment of the present invention, it is possible to obtain a ladder-like amplification product utilizing a nucleotide sequence inherently present in a nucleic acid as a template. In this case, it is

possible to carry out ladder formation utilizing the nucleotide sequence in the nucleic acid as a template. Although it is not intended to limit the present invention, for example, if a nucleotide sequence on the 5' side of or upstream of one of chimeric oligonucleotide primers is complementary to a nucleotide sequence on the 5' side of or upstream of the other chimeric oligonucleotide primer, a ladder-like amplification product can be efficiently obtained by designing the chimeric oligonucleotide primers for such positions. Detection sensitivity and detection velocity are increased by carrying out ladder formation.

[0069]

The complementary nucleotide sequences in the nucleic acid as a template on the 5' sides of or upstream of the chimeric oligonucleotide primers can be arbitrarily selected depending on the GC contents, the adjacent sequences, the distances from the primers, the lengths to be amplified, the reaction conditions and the like. Although it is not intended to limit the present invention, for example, they are preferably selected from complementary nucleotide sequences of 3 nucleotides or more, preferably 6 nucleotides or more. Furthermore, although it is not intended to limit the present invention, the sequences is for example a region from +20 to -100, preferably from +15 to -80, more preferably from +10 to -60,

still more preferably from +5 to -40, defining the 5' terminus of the chimeric oligonucleotide primer as position 0.

The complementary nucleotide sequences in the nucleic acid as a template on the 5' sides of or upstream of the chimeric oligonucleotide primers can be arbitrarily selected depending on the GC contents, the adjacent sequences, the distances from the primers, the lengths to be amplified, the reaction conditions and the like. Although it is not intended to limit the present invention, the complementarities are preferably 70% or more.

[0070]

Although it is not intended to limit the present invention, the ladder-forming oligonucleotide primer used in the method of the present invention is described referring, as examples, to the ladder-forming oligonucleotide primers used in Examples below.

[0071]

For example, in A12-205R (SEQ ID NO:3), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a region 1 to 12 nucleotides upstream of the 5' terminus (-1 to -12) of a second chimeric oligonucleotide primer 134FN3(18) (SEQ ID NO:7), and the nucleotides at positions 13 to 30 constitute a sequence complementary to the region complementary to a

first chimeric oligonucleotide primer 205RN3(18) (SEQ ID NO:2). In other words, the nucleotides at positions 13 to 30 constitute a sequence homologous to the first chimeric oligonucleotide primer.

[0072]

For example, in A12-215R (SEQ ID NO:5), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a region 1 to 12 nucleotides upstream of the 5' terminus (-1 to -12) of a second chimeric oligonucleotide primer 134FN3(18) (SEQ ID NO:7), and the nucleotides at positions 13 to 30 constitute a region complementary to a 8-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer 205RN3(18) (SEQ ID NO:2) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 13 to 30 constitute a sequence homologous to a region starting from the 10th upstream nucleotide to the 8-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides at positions 23 to 30 of A12-215R overlap the nucleotides at positions 1 to 8 of 205RN3(18).

[0073]

For example, in A12-223R (SEQ ID NO:5), the nucleotides at positions 1 to 12 constitute a nucleotide

sequence complementary to a region 1 to 12 nucleotides upstream of the 5' terminus (-1 to -12) of a second chimeric oligonucleotide primer 134FN3(18) (SEQ ID NO:7), and the nucleotides at positions 13 to 30 constitute a sequence complementary to a 18-nucleotide sequence 3' to the region complementary to a first chimeric oligonucleotide primer 205RN3(18) (SEQ ID NO:2). In other words, the nucleotides at positions 13 to 30 constitute a sequence homologous to a sequence from the 18th upstream nucleotide to the 1st upstream nucleotide of the first chimeric oligonucleotide primer. A12-223R is adjacent to 205RN3(18).

[0074]

For example, in A12(-10)-215R (SEQ ID NO:8), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a region 11 to 22 nucleotides upstream of the 5' terminus (-11 to -22) of a second chimeric oligonucleotide primer 134FN3(18) (SEQ ID NO:7), and the nucleotides at positions 13 to 30 constitute a region complementary to a 8-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer 205RN3(18) (SEQ ID NO:2) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 13 to 30 constitute a sequence

homologous to a region starting from the 10th upstream nucleotide to the 8-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides at positions 23 to 30 of A12(-10)-215R overlap the nucleotides at positions 1 to 8 of 205RN3(18).

[0075]

For example, in A12(-20)-215R (SEQ ID NO:9), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a region 21 to 32 nucleotides upstream of the 5' terminus (-21 to -32) of a second chimeric oligonucleotide primer 134FN3(18) (SEQ ID NO:7), and the nucleotides at positions 13 to 30 constitute a region complementary to a 8-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer 205RN3(18) (SEQ ID NO:2) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 13 to 30 constitute a sequence homologous to a region starting from the 10th upstream nucleotide to the 8-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides at positions 23 to 30 of A12(-20)-215R overlap the nucleotides at positions 1 to 8 of 205RN3(18).

[0076]

For example, in A12(6)-215R (SEQ ID NO:10), the

nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a 6-nucleotide sequence on the 5' side (+5 to 0) and a region 1 to 6 nucleotides upstream of the 5' terminus (-1 to -6) of a second chimeric oligonucleotide primer 134FN3(18) (SEQ ID NO:7), and the nucleotides at positions 13 to 30 constitute a region complementary to a 8-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer 205RN3(18) (SEQ ID NO:2) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 13 to 30 constitute a sequence homologous to a region starting from the 10th upstream nucleotide to the 8-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides at positions 23 to 30 of A12(6)-215R overlap the nucleotides at positions 1 to 8 of 205RN3(18).

[0077]

For example, in A12(12)-215R (SEQ ID NO:11), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a 12-nucleotide sequence on the 5' side (+11 to 0) of a second chimeric oligonucleotide primer 134FN3(18) (SEQ ID NO:7), and the nucleotides at positions 13 to 30 constitute a region complementary to a 8-nucleotide sequence on the 5' side of a first chimeric

oligonucleotide primer 205RN3(18) (SEQ ID NO:2) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 13 to 30 constitute a sequence homologous to a region starting from the 10th upstream nucleotide to the 8-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides at positions 23 to 30 of A12(12)-215R overlap the nucleotides at positions 1 to 8 of 205RN3(18).

[0078]

For example, in A6(-10)-215R (SEQ ID NO:14), the nucleotides at positions 1 to 6 constitute a nucleotide sequence complementary to a region 11 to 16 nucleotides upstream of the 5' terminus (-11 to -16) of a second chimeric oligonucleotide primer 134FN3(18) (SEQ ID NO:7), and the nucleotides at positions 7 to 24 constitute a region complementary to a 8-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer 205RN3(18) (SEQ ID NO:2) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 7 to 24 constitute a sequence homologous to a region starting from the 10th upstream nucleotide to the 8-nucleotide sequence on the 5' side of

the first chimeric oligonucleotide primer. The nucleotides at positions 17 to 24 of A6(-10)-215R overlap the nucleotides at positions 1 to 8 of 205RN3(18).

[0079]

For example, in A9(-10)-215R (SEQ ID NO:15), the nucleotides at positions 1 to 9 constitute a nucleotide sequence complementary to a region 11 to 19 nucleotides upstream of the 5' terminus (-11 to -19) of a second chimeric oligonucleotide primer 134FN3(18) (SEQ ID NO:7), and the nucleotides at positions 10 to 27 constitute a region complementary to a 8-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer 205RN3(18) (SEQ ID NO:2) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 10 to 27 constitute a sequence homologous to a region starting from the 10th upstream nucleotide to the 8-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides at positions 20 to 27 of A9(-10)-215R overlap the nucleotides at positions 1 to 8 of 205RN3(18).

[0080]

For example, in (A12)241RN3 (SEQ ID NO:19), the nucleotides at positions 1 to 7 constitute a nucleotide sequence complementary to a region 1 to 7 nucleotides

upstream of the 5' terminus (-1 to -7) of a second chimeric oligonucleotide primer 160FN3 (SEQ ID NO:17), and the nucleotides at positions 8 to 21 constitute a sequence complementary to the region complementary to a first chimeric oligonucleotide primer 241RN3 (SEQ ID NO:18). In other words, the nucleotides at positions 8 to 21 constitute a sequence homologous to the first chimeric oligonucleotide primer.

[0081]

For example, in ALDH2-TH1 (SEQ ID NO:25), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a region 1 to 12 nucleotides upstream of the 5' terminus (-1 to -12) of a second chimeric oligonucleotide primer ICAN-ALDH2-F (SEQ ID NO:21), and the nucleotides at positions 13 to 29 constitute a region of a nucleic acid as a template complementary to a 15-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer ICAN-ALDH2-R (SEQ ID NO:22) and a sequence complementary to a 2-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, they constitute a sequence homologous to a region starting from the 2nd upstream nucleotide to the 15-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides at positions 2 to 16 of ICAN-ALDH2-R overlap the

nucleotides at positions 15 to 29 of ALDH2-TH1.

[0082]

For example, in ALDH2-TH2 (SEQ ID NO:26), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a region 1 to 12 nucleotides upstream of the 5' terminus (-1 to -12) of a second chimeric oligonucleotide primer ICAN-ALDH2-F (SEQ ID NO:21), and the nucleotides at positions 13 to 29 constitute a region complementary to a 6-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer ICAN-ALDH2-R (SEQ ID NO:22) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 13 to 29 constitute a sequence homologous to a region starting from the 10th upstream nucleotide to the 6-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides at positions 2 to 7 of ICAN-ALDH2-R overlap the nucleotides at positions 23 to 28 of ALDH2-TH2.

[0083]

For example, in ALDH2-TH3 (SEQ ID NO:27), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a region 1 to 12 nucleotides upstream of the 5' terminus (-1 to -12) of a second chimeric oligonucleotide primer ICAN-ALDH2-F (SEQ ID NO:21),

and the nucleotides at positions 13 to 28 constitute a sequence complementary to a nucleotide sequence 2 to 17 nucleotides 3' to the sequence complementary to a first chimeric oligonucleotide primer ICAN-ALDH2-R (SEQ ID NO:22). In other words, the nucleotides at positions 13 to 28 constitute a sequence homologous to a sequence 2 to 17 nucleotides upstream of the first chimeric oligonucleotide primer.

[0084]

For example, in R2(-13)A12-1 (SEQ ID NO:35), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a region 1 to 12 nucleotides upstream of the 5' terminus (-1 to -12) of a second chimeric oligonucleotide primer F2 (SEQ ID NO:31), and the nucleotides at positions 13 to 29 constitute a sequence complementary to a nucleotide sequence 13 to 29 nucleotides 3' to the sequence complementary to a first chimeric oligonucleotide primer R2 (SEQ ID NO:32). In other words, the nucleotides at positions 13 to 29 constitute a sequence homologous to a sequence 13 to 29 nucleotides upstream of the first chimeric oligonucleotide primer.

[0085]

For example, in R2(-13)A12-2 (SEQ ID NO:36), the nucleotides at positions 1 to 12 constitute a nucleotide sequence complementary to a region 13 to 24 nucleotides

upstream of the 5' terminus (-13 to -24) of a second chimeric oligonucleotide primer F2 (SEQ ID NO:31), and the nucleotides at positions 13 to 29 constitute a sequence complementary to a nucleotide sequence 13 to 29 nucleotides 3' to the sequence complementary to a first chimeric oligonucleotide primer R2 (SEQ ID NO:32). In other words, the nucleotides at positions 13 to 29 constitute a sequence homologous to a sequence 13 to 29 nucleotides upstream of the first chimeric oligonucleotide primer.

[0086]

For example, in A6-215R (SEQ ID NO:47), the nucleotides at positions 1 to 6 constitute a nucleotide sequence complementary to a region 1 to 6 nucleotides upstream of the 5' terminus (-1 to -6) of a second chimeric oligonucleotide primer B134FN3 (SEQ ID NO:12), and the nucleotides at positions 7 to 24 constitute a region complementary to a 8-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer 205RN3(16) (SEQ ID NO:13) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 7 to 24 constitute a sequence homologous to a region starting from the 10th upstream nucleotide to the 8-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides

at positions 1 to 8 of 205RN3(16) overlap the nucleotides at positions 17 to 24 of A6-215R.

[0087]

For example, in A9-215R (SEQ ID NO:48), the nucleotides at positions 1 to 9 constitute a nucleotide sequence complementary to a region 1 to 9 nucleotides upstream of the 5' terminus (-1 to -9) of a second chimeric oligonucleotide primer B134FN3 (SEQ ID NO:12), and the nucleotides at positions 10 to 27 constitute a region complementary to a 8-nucleotide sequence on the 5' side of a first chimeric oligonucleotide primer 205RN3(16) (SEQ ID NO:13) and a sequence complementary to a 10-nucleotide sequence 3' to the region complementary to the first chimeric oligonucleotide primer. In other words, the nucleotides at positions 7 to 24 constitute a sequence homologous to a region starting from the 10th upstream nucleotide to the 8-nucleotide sequence on the 5' side of the first chimeric oligonucleotide primer. The nucleotides at positions 1 to 8 of 205RN3(16) overlap the nucleotides at positions 20 to 27 of A6-215R.

[0088]

Positional relationships among the first chimeric oligonucleotide primer, the second chimeric oligonucleotide primer and the ladder-forming oligonucleotide primers used in Example 2-(1)-(A) are illustrated in Figure 1.

[0089]

Any RNase H can be preferably used in the method of the present invention, including mesophilic and heat-resistant ones. For example, RNase HII from *Thermococcus litralis* (hereinafter referred to as Tli RNase HII) prepared according to the method as described in Example 8 of WO 02/22831 or RNase HII from *Archaeoglobus fulgidus* (hereinafter referred to as Afu RNase HII) prepared according to the method as described in Example 7 of WO 02/22831 can be used in the method of the present invention as described in Examples below. Examples of heat-resistant RNase Hs that can be preferably used include, but are not limited to, a commercially available RNase H, Hybridase™ Thermostable RNase H (Epicentre Biotechnologies) as well as an RNase H from a thermophilic bacterium of the genus *Bacillus*, a bacterium of the genus *Thermus*, a bacterium of the genus *Pyrococcus*, a bacterium of the genus *Thermotoga*, a bacterium of the genus *Archaeoglobus* or the like. Furthermore, both of naturally occurring RNase Hs and variants can be preferably used.

[0090]

Any DNA polymerases having the strand displacement activity can be used according to the present invention. Examples thereof include variants of DNA polymerases lacking their 5'→3' exonuclease activities

derived from thermophilic bacteria of the genus *Bacillus* such as *Bacillus caldotenax* (hereinafter referred to as B. ca) and *Bacillus stearothermophilus* (hereinafter referred to as B. st), as well as large fragment (Klenow fragment) of DNA polymerase I from *Escherichia coli* (*E. coli*). Both of mesophilic and heat-resistant DNA polymerases can be preferably used according to the present invention.

B. ca is a thermophilic bacterium having an optimal growth temperature of about 70°C. Bca DNA polymerase from this bacterium is known to have a DNA-dependent DNA polymerase activity, an RNA-dependent DNA polymerase activity (a reverse transcription activity), a 5'→3' exonuclease activity and a 3'→5' exonuclease activity. The enzyme may be either an enzyme purified from its original source or a recombinant protein produced by using genetic engineering techniques. The enzyme may be subjected to modification such as substitution, deletion, addition or insertion by using genetic engineering techniques or other means. Examples of such enzymes include BcaBEST DNA polymerase (Takara Bio), which is Bca DNA polymerase lacking its 5'→3' exonuclease activity. This enzyme can be prepared according to the method as described in Japanese Patent No. 2978001 from the microorganism disclosed therein.

[0091]

It is known that a certain DNA polymerase has an endonuclease activity such as an RNase H activity under specific conditions. Such a DNA polymerase can be used in the method of the present invention. In one aspect, the DNA polymerase may be used under conditions under which an RNase H activity is exhibited, e.g., in the presence of $Mn^{2+}$. In this case, the method of the present invention can be conducted without the addition of an RNase H. Thus, the Bca DNA polymerase can exhibit an RNase activity in a buffer containing $Mn^{2+}$. The above-mentioned aspect is not limited to the use of the Bca DNA polymerase. A DNA polymerase known to additionally have an RNase H activity such as Tth DNA polymerase from *Thermus thermophilus* can be used according to the present invention.

[0092]

A ladder-forming oligonucleotide primer may be used in the method of the present invention; a first chimeric oligonucleotide primer may also function as a ladder-forming oligonucleotide primer; or a ladder-like amplification product may be obtained utilizing a complementary nucleotide sequence in a nucleic acid as a template. In either case, it is possible to stabilize the reaction by conducting the reaction in the presence of a random primer to efficiently obtain the amplification product of interest. Although there is no specific

limitation concerning the random primer, for example, a random primer of 6 to 9 nucleotides in length can be preferably used.

[0093]

(2) The composition of the present invention

The present invention provides a composition used for the method for amplifying a nucleic acid of the present invention or the method for detecting a nucleic acid of the present invention as described above. For example, the composition may contain a ladder-forming oligonucleotide primer and/or a chimeric oligonucleotide primer, an RNase H and a DNA polymerase as described in (1) above. The composition may further contain a buffering component, a magnesium salt, dNTPs and the like as components for conducting an amplification reaction. Furthermore, it may contain a modified deoxyribonucleotide or a deoxynucleotide triphosphate analog. Additionally, a buffering component and other additives can be used.

[0094]

In a particularly preferable aspect, the composition may contain suitable amounts of the various components as listed above for the nucleic acid amplification method of the present invention. An amplification reaction can be conducted only by adding an appropriate template, a chimeric oligonucleotide primer

and/or a ladder-forming oligonucleotide to the composition. If the subject to be amplified is known beforehand, the composition preferably contains a chimeric oligonucleotide primer suitable for the amplification of the subject.

[0095]

(3) The kit of the present invention

The present invention provides a kit used for the method for amplifying a nucleic acid of present invention as described above. In one embodiment, the kit is in a packed form and contains instructions regarding the use of a DNA polymerase, an RNase H, a chimeric oligonucleotide primer and/or a ladder-forming oligonucleotide primer in a strand displacement reaction. Also, a kit that contains a DNA polymerase having a strand displacement activity, an RNase H, and a buffer for a strand displacement reaction is preferably used for the method of the present invention. Alternatively, a commercially available DNA polymerase having a strand displacement activity and/or RNase H may be selected and used according to the instructions. Additionally, the kit may contain a reagent for a reverse transcription reaction which is used if an RNA is to be used as a template. The DNA polymerase can be selected from the DNA polymerases to be used according to the present invention as described in (1) above. The RNase H can be selected from the RNase Hs as described in (1) above.

[0096]

"Instructions" are printed matters describing a method of using the kit, e.g., a method of preparing a reagent solution for a strand displacement reaction, recommended reaction conditions and the like. The instructions include an instruction manual in a form of a pamphlet or a leaflet, a label stuck to the kit, and description on the surface of the package containing the kit. The instructions also include information disclosed or provided through electronic media such as the Internet.

[0097]

The kit of the present invention may further contain a reaction buffer containing Bicine, Tricine, HEPES, phosphate or tris-hydrochloride as a buffering component and an annealing solution. Additionally, it may contain a DNA polymerase having a strand displacement activity and an RNase H. Furthermore, the kit may contain a modified deoxyribonucleotide or a deoxynucleotide triphosphate analog.

[0098]

The kit used in the method for detecting a target nucleic acid may further contain a chimeric oligonucleotide primer and/or a ladder-forming oligonucleotide primer suitable for amplification of a target nucleic acid, and a reagent for detecting the amplified target nucleic acid

(e.g., a probe) in addition to the instructions and the reagents for amplification reaction as described above.

[0099]

(4) The method for detecting a target nucleic acid of the present invention

It is possible to detect a target nucleic acid in a sample using the method for amplifying a nucleic acid of the present invention. In one embodiment, the detection method comprises the steps of:

(a) amplifying a target nucleic acid according to the method for amplifying a nucleic acid of the present invention as described in (1) above; and

(b) detecting the target nucleic acid amplified in the above step.

[0100]

If an RNA is to be used as a template in step (a) above, the reverse transcription reaction and the nucleic acid amplification reaction may be conducted in one or two step(s). Although it is not intended to limit the present invention, for example, a combination of AMV RTase, M-MLV RTase or RAV-2 RTase and BcaBEST DNA polymerase can be preferably used as a combination of a reverse transcriptase and a strand displacement-type DNA polymerase. Alternatively, a DNA polymerase having a reverse transcription activity and a strand displacement activity

may be used. For example, BcaBEST DNA polymerase can be preferably used.

[0101]

Information about a specific nucleotide in a gene such as a point mutation or a single nucleotide polymorphism (SNP) can be obtained using the method for detecting a target nucleic acid of the present invention. In this embodiment, the 3'-terminal portion of the chimeric oligonucleotide primer to be used may be placed in the vicinity of the specific nucleotide in the target nucleotide sequence to be distinguished.

[0102]

In addition, a target nucleic acid can be detected with higher sensitivity even from a trace amount of a nucleic acid sample according to the detection method of the present invention by using a reaction buffer containing Bicine, Tricine, HEPES, phosphate or tris as a buffering component and an annealing solution containing spermidine or propylenediamine. In this case, the RNase H and the DNA polymerase to be used are not limited to specific ones. For example, a combination of RNase HII from a bacterium of the genus *Thermococcus* and BcaBEST DNA polymerase is preferable. It is considered that the preferable unit numbers of the RNase H and the DNA polymerase may vary depending on the types of the enzymes.

In such a case, the composition of the buffer and the amounts of the enzymes to be added may be adjusted using the increase in detection sensitivity or the amount of amplification product as an index.

[0103]

According to the detection method of the present invention, dUTP may be incorporated as a substrate during amplification of a target nucleic acid. Thus, if dUTP is used as a substrate, it is possible to prevent false positives due to carry-over contamination of amplification products by degrading amplification products utilizing uracil N-glycosidase (UNG).

[0104]

A known method for detecting a nucleic acid can be used for step (b). For example, detection of a reaction product having a specific size by electrophoresis, real-time detection using Smart Cycler (Takara Bio), Rotor gene (Corbett Research) or the like, or detection by hybridization with a probe can be used. Furthermore, a detection method in which magnetic beads are used in combination can be preferably used. The reaction mixture may be made turbid by converting pyrophosphoric acid generated during the step of amplification of a target nucleic acid into a insoluble substance such as a magnesium salt, and then the turbidity may be measured.

A fluorescent substance such as ethidium bromide is usually used for the detection by electrophoresis. The hybridization with a probe may be combined with the detection by electrophoresis. The probe may be labeled with a radioisotope or with a non-radioactive substance such as biotin or a fluorescent substance. If real-time detection is to be carried out, a coloring reagent such as SYBR Green (Takara Bio) can be preferably used. Additionally, use of a labeled nucleotide in step (a) may facilitate the detection of amplification product into which the labeled nucleotide is incorporated, or may enhance the signal for detection utilizing the label. A fluorescence polarization method, a fluorescence energy transition or the like can also be utilized for the detection. The target nucleic acid can be detected automatically or quantified by constructing a suitable detection system. In addition, detection with naked eyes using a hybrid chromatography method can be preferably used.

[0105]

A ribonucleotide (RNA) probe, or a chimeric oligonucleotide probe composed of ribonucleotides and deoxyribonucleotides, labeled with two or more fluorescent substances positioned at a distance that results in a quenching state can be used in the detection method of the present invention. The probe does not emit fluorescence.

When it is annealed to a DNA amplified from a target nucleic acid that is complementary to the probe, RNase H digests the probe. The distance between the fluorescent substances on the probe then increases, resulting in the emission of fluorescence. Thus, the emission reveals the presence of the target nucleic acid. If RNase H is used in the method for amplifying a nucleic acid of the present invention, a target nucleic acid can be detected only by adding the probe to the reaction mixture. For example, a combination of a fluorescent substance ROX (Applied Biosystems) or FAM (Applied Biosystems) and a quenching substance Eclipse (Epoch Biosciences) can be preferably used for labeling the probe.

[0106]

The probe used according to the present invention is not limited to specific one as long as it can hybridize to a target nucleic acid amplified according to the nucleic acid amplification method of the present invention under normal hybridization conditions. In view of specific detection of amplification product, a probe that hybridizes under conditions, for example, known to those skilled in the art as being stringent is preferable. The stringent hybridization conditions are described in, for example, T. Maniatis et al. (eds.), Molecular Cloning: A Laboratory Manual 2nd ed., 1989, Cold Spring Harbor Laboratory.

Specifically, the stringent conditions are exemplified by the following: incubation at a temperature about 25°C lower than the Tm value of the probe to be used for 4 hours to overnight in 6 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.5% SDS, 5 x Denhardt's (0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400) and 100 µg/ml salmon sperm DNA. A probe having a label as described above may be used as the probe for facilitating the detection of the target nucleic acid.

[0107]

A ladder-like amplification product in which amplified regions are connected to each other is positively generated according to the method for detecting a target nucleic acid of the present invention. In the ladder-like amplification product, plural amplified regions are polymerized through an arbitrary nucleotide sequence in the same direction. The amplification product is observed as a ladder-like band upon analysis of the amplification product by electrophoresis. The generation of the ladder-like amplification product can be adjusted depending on the region to be amplified, the size of the region, the adjacent region, the nucleotide sequence of the chimeric oligonucleotide primer and/or the ladder-forming oligonucleotide primer to be used, the reaction conditions or the like.

[0108]

According to the detection method of the present invention, the ladder-forming oligonucleotide primer and/or the chimeric oligonucleotide primer can be used to generate a polymer in which amplified regions are connected to each other. In the ladder-like amplification product, plural amplified regions are connected to each other in the same direction through the ladder-forming oligonucleotide primer and a sequence complementary to a region on the 5' side of or upstream of a second chimeric oligonucleotide primer. Furthermore, according to the method of the present invention, if a nucleotide sequence of a template nucleic acid on the 5' side of or upstream of one of chimeric oligonucleotide primers is complementary to a nucleotide sequence on the 5' side of or upstream of the other chimeric oligonucleotide primer, a target nucleic acid can be detected with high sensitivity by designing the chimeric oligonucleotide primers for the portions.

[0109]

The ladder-like amplification product contains plural amplified regions. Therefore, it can be hybridized with many copies of an appropriate probe, for example. One that hybridizes to the nucleotide sequence of the target nucleic acid and/or the intervening sequence existing between the target nucleic acid sequences in the ladder-

like amplification product can be preferably used as such a probe. Since an intense signal is consequently emitted, the present invention is useful if a nucleic acid containing an amplified region is to be detected. It is possible to obtain an amplified region or a part thereof as a monomer from a ladder-like amplification product by using restriction enzyme digestion or the like in combination.

[0110]

The isothermal nucleic acid amplification method of the present invention does not require the use of equipment such as a thermal cycler. Since reagents such as dNTPs used for PCR and the like can be applied to the method, the running cost can be reduced as compared with a conventional method. Therefore, the method of the present invention can be preferably used, for example, in a field of genetic test in which the detection is routinely conducted. The method of the present invention provides a greater amount of an amplification product in a shorter time than the PCR. Therefore, the method of the present invention can be utilized as a convenient, rapid and sensitive gene detection method.

Examples

[0111]

The following examples further illustrate the

present invention in detail but are not to be construed to limit the scope thereof.

[0112]

Referential Example 1

A unit number of a heat-resistant RNase H in Examples was calculated as follows.

1 mg of poly(rA) or poly(dT) (both from Amersham Biotech) was dissolved in 1 ml of 40 mM tris-HCl (pH 7.7) containing 1 mM EDTA to prepare a poly(rA) solution and a poly(dT) solution.

The poly(rA) solution (to a final concentration of 30 µg/ml) and the poly(dT) solution (to a final concentration of 20 µg/ml) were then added to 20 mM HEPES-KOH (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide and 0.01% BSA. The mixture was incubated at 37°C for 10 minutes and then cooled to room temperature. 1/100 volume of 400 mM magnesium acetate was added thereto to prepare a poly(rA)-poly(dT) solution.

8 µl of an appropriate dilution of an enzyme solution was added to 800 µl of the poly(rA)-poly(dT) solution. The mixture was reacted at 40°C for 20 minutes. The absorbance (A260) was measured over time to determine the change. Specifically, the concentration of nucleotide released from poly(rA)-poly(dT) hybrid by the enzymatic reaction was determined on the basis of the difference in

absorbance. One unit of an RNase H was defined as an amount of enzyme that increases A260 corresponding to release of 1 nmol of ribonucleotide in 20 minutes calculated according to the following equation:

Unit = Change in absorbance per minute x 57.1 x Dilution rate

[0113]

Example 1

(1) Preparation of template RNA

An RNA transcript used as a template for RT-ICAN was synthesized by in vitro transcription.

A plasmid DNA to be used as a template for in vitro transcription was synthesized by artificial synthetic gene preparation commission service (Takara Bio). A sequence from position 18133 to position 18362 (SEQ ID NO:1) of the genomic sequence of SARS (Severe Acute Respiratory Syndrome) coronavirus (GenBank Acc. No.: AY278741) was used. Recognition sequences for HindIII and BamHI for insertion into a plasmid were added at the 5' terminus and the 3' terminus of the sequence, respectively. This fragment was inserted between the HindIII site and the BamHI site in pBluescriptII SK(+). The resulting plasmid was cleaved with BamHI to obtain a linear DNA, which was used as a template for in vitro transcription using T7 RNA polymerase.

MEGAscript T7 Kit (Ambion) was used for in vitro transcription. In vitro transcription, DNase I treatment and RNA purification were carried out according to the instructions attached to the kit, and the concentration of the synthesized RNA was measured on the basis of OD260. The RNA solution whose concentration was adjusted to $10^0$ to $10^5$ copies/μl with RNA Dilution Buffer (10 mM Tris-HCl (pH7.5), 1 mM EDTA, 10 mM NaCl, 30 μg/ml *E. coli* 16S+23S rRNA (Boehringer Ingerheim)) was used as a template for RT-ICAN.

[0114]

(2) Examination of 2-step RT-ICAN

(A) Examination 1

The effect of attachment, at the 5' terminus of a reverse transcription primer, of a sequence that anneals to a region upstream of a second chimeric oligonucleotide primer was examined.

[0115]

Changes in sensitivity and detection velocity of RT-ICAN by the use, as a reverse transcription primer, of a ladder-forming oligonucleotide primer having a 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -12) of a second chimeric oligonucleotide primer were studied.

The concentration of the template RNA prepared in

Example 1-(1) was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ copies per µl. A reaction mixture of 10 µl containing the following at final concentrations was prepared: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 1 pmol of a primer 205RN3(18) (SEQ ID NO:2), a primer A12-205R (SEQ ID NO:3), a primer 215R (SEQ ID NO:4), a primer A12-215R (SEQ ID NO:5) or a primer A12-223R (SEQ ID NO:6) as a reverse transcription primer, 12.5 U of RTase M-MLV (Takara Bio) and 1 µl of one of the template RNAs with varying copy numbers.

[0116]

The reaction mixture was placed in Thermal Cycler Personal (Takara Bio), incubated at 45°C for 10 minutes, and then cooled to 4°C. After reverse transcription reaction, 15 µl of a reaction mixture containing the following at final concentrations was added to 10 µl of the reaction mixture: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 25 pmol each of a primer 134FN3(18) (SEQ ID NO:7) as a second chimeric oligonucleotide primer and a primer 205RN3(18) as a first chimeric oligonucleotide primer, 11 U of BcaBEST (Takara Bio), 0.05 U of Tli RNase HII prepared according to the

method as described in Example 8 of WO 02/22831 and SYBR Green (Takara Bio). An ICAN reaction was carried out at 55°C using Rotor gene (Corbett Research) to detect the amplification product in a real-time manner. The reaction product was also detected by electrophoresis on 3% agarose gel.

[0117]

As a result, the sensitivity of RT-ICAN with reverse transcription using the primer 205RN3(18) was $10^4$ copies. When the reverse transcription primer A12-205R for the same position having a 12-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -12) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^2$ copies, i.e., increased by two orders of magnitude. In addition, the detection velocity for $10^5$ copies was increased by about 2 minutes.

[0118]

Similarly, the sensitivity of RT-ICAN with reverse transcription using the primer 215R DNA was $10^3$ copies. When the ladder-forming oligonucleotide primer A12-215R for the same position having a 12-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -12) of the second chimeric oligonucleotide primer was used for reverse transcription, the sensitivity was $10^0$ copy, i.e., increased by three

orders of magnitude. In addition, the detection velocity for $10^5$ copies was increased by about 2 minutes.

[0119]

Furthermore, when the ladder-forming oligonucleotide primer A12-223R having a 12-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -12) of the second chimeric oligonucleotide primer was used for reverse transcription, the sensitivity was $10^1$ copies.

[0120]

As described above, increases in sensitivity and detection velocity were observed when a ladder-forming oligonucleotide primer having a 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -12) of a second chimeric oligonucleotide primer was used for reverse transcription, regardless of the portion of the template RNA to which the primer anneals.

[0121]

(B) Examination 2

Similar examination was carried out using a ladder-forming oligonucleotide primer as a reverse transcription primer while changing the position of a 12-nucleotide sequence (-1 to -12, -11 to -22, -21 to -32, +5 to -6, +11 to 0) attached at the 5' terminus that anneals to a region upstream of a second chimeric oligonucleotide

primer and/or a part thereof.

First, the concentration of the template RNA prepared in Example 1-(1) was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ copies per µl.

A reaction mixture of 10 µl containing the following at final concentrations was prepared: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 1 pmol of a ladder-forming oligonucleotide primer, a primer 215R, a primer A12-215R, a primer A12(-10)-215R (SEQ ID NO:8), a primer A12(-20)-215R (SEQ ID NO:9), a primer A12(6)-215R (SEQ ID NO:10) or a primer A12(12)-215R (SEQ ID NO:11), as a reverse transcription primer, 50 U of RTase M-MLV (Takara Bio) and 1 µl of one of the template RNAs with varying copy numbers.

[0122]

The reaction mixture was placed in Thermal Cycler Personal (Takara Bio), incubated at 45°C for 10 minutes, and then cooled to 4°C. After reverse transcription reaction, 15 µl of a reaction mixture containing the following at final concentrations was added to 10 µl of the reaction mixture: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 25 pmol each of a

primer B134FN3(16) (SEQ ID NO:12) as a second chimeric oligonucleotide primer and a primer 205RN3(16) (SEQ ID NO:13) as a first chimeric oligonucleotide primer, 11 U of BcaBEST (Takara Bio), 0.05 U of Tli RNase HII and SYBR Green (Takara Bio). An ICAN reaction was carried out at 55°C using Rotor gene (Corbett Research) to detect the amplification product in a real-time manner. The reaction product was also detected by electrophoresis on 3% agarose gel.

[0123]

As a result, the sensitivity of RT-ICAN with reverse transcription using the primer 215R was $10^2$ copies. When the ladder-forming oligonucleotide primer A12-215R for the same position having a 12-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -12) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^1$ copies, i.e., increased by one order of magnitude. In addition, the detection velocity for $10^5$ copies was increased by about 3 minutes.

[0124]

When the ladder-forming oligonucleotide primer A12(-10)-215R having a 12-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-11 to -22) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^2$ copies, which was the same as that

with the primer 215R. The detection velocity for $10^5$ copies was increased by about 2 minutes. The detection of $10^2$ to $10^5$ copies was more quantitative (correlation coefficient: 0.997) than that with the primer 215R.

[0125]

When the ladder-forming oligonucleotide primer A12(-20)-215R having a 12-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-21 to -32) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^1$ copies, i.e., increased by one order of magnitude. In addition, the detection velocity for $10^5$ copies was increased by about 2.5 minutes.

[0126]

When the ladder-forming oligonucleotide primer A12(6)-215R having a 12-nucleotide sequence attached at the 5' terminus that anneals to the upstream region and a part of the second chimeric oligonucleotide primer, or a ladder-forming oligonucleotide primer A12(12)-215R having a 12-nucleotide sequence attached at the 5' terminus that anneals to a part of the second chimeric oligonucleotide primer was used, increases in detection sensitivity and detection velocity were also observed like the case of the above-mentioned primer.

[0127]

As described above, increases in sensitivity and

detection velocity were observed using each ladder-forming oligonucleotide primer while changing the position of a 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream of a second chimeric oligonucleotide primer.

[0128]

(C) Examination 3

Sensitivity and detection velocity of RT-ICAN were examined using a ladder-forming oligonucleotide primer having a 6-, 9- or 12-nucleotide sequence attached at the 5' terminus (-11 to -16, -11 to -19 or -11 to -22) that anneals to a region upstream of a second chimeric oligonucleotide primer as a reverse transcription primer.

First, the concentration of the template RNA prepared in Example 1-(1) was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ copies per µl.

A reaction mixture of 10 µl containing the following at final concentrations was prepared: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 1 pmol of a ladder-forming oligonucleotide primer, a primer 215R, a primer A6(-10)-215R (SEQ ID NO:14), a primer A9(-10)-215R (SEQ ID NO:15) or a primer A12(-10)-215R, as a reverse transcription

primer, 50 U of RTase M-MLV (Takara Bio) and 1 µl of one of the template RNAs with varying copy numbers.

[0129]

The reaction mixture was placed in Thermal Cycler Personal (Takara Bio), incubated at 45°C for 10 minutes, and then cooled to 4°C. After reverse transcription reaction, 15 µl of a reaction mixture containing the following at final concentrations was added to 10 µl of the reaction mixture: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 25 pmol each of a primer B134FN3(16) as a second chimeric oligonucleotide primer and a primer 205RN3(16) as a first chimeric oligonucleotide primer, 11 U of BcaBEST (Takara Bio), 0.05 U of Tli RNase HII and SYBR Green. An ICAN reaction was carried out at 55°C using Rotor gene (Corbett Research) to detect the amplification product in a real-time manner. The reaction product was also detected by electrophoresis on 3% agarose gel.

[0130]

As a result, the sensitivity of RT-ICAN with reverse transcription using the primer 215R was $10^3$ copies. When the ladder-forming oligonucleotide primer A6(-10)-215R for the same position having a 6-nucleotide sequence attached at the 5' terminus that anneals to the region

upstream (-11 to -16) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^2$ copies, i.e., increased by one order of magnitude. In addition, the detection velocity for $10^5$ copies was increased by about 1 minute.

[0131]

When the ladder-forming oligonucleotide primer A9(-10)-215R having a 9-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-11 to -19) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^1$ copies, i.e., increased by two orders of magnitude. In addition, the detection velocity for $10^5$ copies was increased by about 2.5 minutes.

[0132]

When the ladder-forming oligonucleotide primer A12(-10)-215R having a 12-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-11 to -22) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^1$ copies, i.e., increased by two orders of magnitude. In addition, the detection velocity for $10^5$ copies was increased by about 3 minutes.

[0133]

As described above, among the ladder-forming oligonucleotide primers with varying numbers of attached nucleotides, increases in sensitivity and detection

velocity were observed using a ladder-forming oligonucleotide primer having a sequence of 6 nucleotides or more attached at the 5' terminus that anneals to a region upstream of a second chimeric oligonucleotide primer. It was confirmed that a sequence of more nucleotides that anneals to a region upstream of a second chimeric oligonucleotide primer results in a more effect.

[0134]

(D) Examination 4

Detection velocity and quantitativeness of RT-ICAN were examined using A12-215R, a ladder-forming oligonucleotide primer having a 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -12) of a second chimeric oligonucleotide primer.

First, the concentration of the template RNA prepared in Example 1-(1) was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ copies per µl.

A reaction mixture of 10 µl containing the following at final concentrations was prepared: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 1 pmol of the primer A12-215R, 50 U of RTase M-MLV (Takara Bio) and 1 µl of one of the template

RNAs with varying copy numbers.

[0135]

The reaction mixture was placed in Thermal Cycler Personal (Takara Bio), incubated at 45°C for 10 minutes, and then cooled to 4°C. After reverse transcription reaction, 15 µl of a reaction mixture containing the following at final concentrations was added to 10 µl of the reaction mixture: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 25 pmol each of a primer B134FN3(16) as a second chimeric oligonucleotide primer and a primer 205RN3(16) as a first chimeric oligonucleotide primer, 11 U of BcaBEST (Takara Bio), 0.05 U of Tli RNase HII and SYBR Green. An ICAN reaction was carried out at 55°C using Rotor gene (Corbett Research) to detect the amplification product in a real-time manner at every cycle (1 minute).

[0136]

Amplification curves are shown in Figure 2. As shown in Figure 2, $10^1$ to $10^5$ copies were quantitatively detected (correlation coefficient: 0.992). Rise of the reaction for down to 10 copies was observed within 10 cycles.

[0137]

Furthermore, amplification products were also

observed according to a probe hybridization method by development on chromatography strips.

[0138]

Observation of amplification products according to a probe hybridization method by development on chromatography strips was carried out using Detection Set contained in TaKaRa Bed-Side ICAN $bla^{IMP}$ Detection Kit (Takara Bio) according to the detection method described in the attached instructions. In the detection, a probe solution containing a FITC-labeled probe SARS-BNI-B (SEQ ID NO:16) was used as a probe, and blaMP Probe Solution and IC Probe Solution contained in the kit were not used.

[0139]

As a result, development of reddish purple lines was observed for the amplification products from the reactions with $10^0$ to $10^5$ copies, indicating specific amplification.

[0140]

(3) Examination of 1-step RT-ICAN

(A) Examination 1

The effect of the use of a first chimeric oligonucleotide primer having a sequence attached at the 5' terminus that anneals to a region upstream of a second chimeric oligonucleotide primer as a ladder-forming oligonucleotide primer was examined.

[0141]

Sensitivity and detection velocity of RT-ICAN were examined using a first chimeric oligonucleotide primer having a 7-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -7) of a second chimeric oligonucleotide primer. As a result of the attachment of the 7-nucleotide sequence at the 5' terminus of the first chimeric oligonucleotide primer, a sequence of 12 nucleotides from the 5' terminus of the first chimeric oligonucleotide primer constituted a sequence that anneals to a region upstream (-1 to -12) of the second chimeric oligonucleotide primer. A reverse transcription reaction and an ICAN reaction were carried out in parallel in the same tube.

First, the concentration of the template RNA prepared in Example 1-(1) was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ copies per µl. 1 µl of one of the template RNAs with varying copy numbers was added to a reaction mixture of 24 µl containing the following at final concentrations: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 25 pmol each of a primer 160FN3 (SEQ ID NO:17) as a second chimeric oligonucleotide primer and a primer 241RN3 (SEQ ID NO:18) as a first chimeric

oligonucleotide primer, or a primer (A12)241RN3 (SEQ ID NO:19) as a first chimeric oligonucleotide primer, 50 U of RTase M-MLV (Takara Bio), 11 U of BcaBEST (Takara Bio), 0.05 U of Tli RNase HII and SYBR Green (Takara Bio). The reaction mixture was incubated at 45°C for 5 minutes and at 55°C for 45 minutes using Rotor gene (Corbett Research) to detect the amplification product in a real-time manner. The reaction product was also detected by electrophoresis on 3% agarose gel.

[0142]

As a result, the sensitivity of RT-ICAN using the primer 241RN3 as a first chimeric oligonucleotide primer was $10^5$ copies. When the primer (A12)241RN3 having a 7-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -7) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^3$ copies, i.e., increased by two orders of magnitude. In addition, the detection velocity for $10^5$ copies was increased by about 1 minute.

[0143]

As described above, increases in sensitivity and detection velocity were observed using a first chimeric oligonucleotide primer having a sequence attached at the 5' terminus that anneals to a region upstream of a second chimeric oligonucleotide primer as a ladder-forming

oligonucleotide primer.

[0144]

(B) Examination 2

The effect, on a 1-step RT-ICAN reaction, of a ladder-forming oligonucleotide primer having a sequence attached at the 5' terminus that anneals to a region upstream of a second chimeric oligonucleotide primer was examined.

[0145]

A ladder-forming oligonucleotide primer having a 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -12) of a second chimeric oligonucleotide primer was added to a 1-step RT-ICAN reaction system in which a reverse transcription reaction and an ICAN reaction were carried out in parallel in the same tube, and the sensitivity and the detection velocity were examined.

First, the concentration of the template RNA prepared in Example 1-(1) was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ copies per µl. 1 µl of one of the template RNAs with varying copy numbers was added to a reaction mixture of 24 µl containing the following at final concentrations: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500

μM each of dNTPs, 25 pmol each of a primer 134FN3(16) (SEQ ID NO:20) as a second chimeric oligonucleotide primer and a primer 205RN3(16) as a first chimeric oligonucleotide primer, 50 U of RTase M-MLV (Takara Bio), 11 U of BcaBEST (Takara Bio), 0.05 U of Tli RNase HII and SYBR Green (Takara Bio), or a reaction mixture of 24 μl further containing 2.5 pmol of a primer A12-223R as a ladder-forming oligonucleotide primer. The reaction mixture was incubated at 45°C for 5 minutes and at 55°C for 45 minutes using Rotor gene (Corbett Research) to detect the amplification product in a real-time manner. The reaction product was also detected by electrophoresis on 3% agarose gel.

[0146]

As a result, the sensitivity of 1-step RT-ICAN without the addition of the primer A12-223R as a ladder-forming oligonucleotide primer was $10^3$ copies. When the primer A12-223R was added, the sensitivity was $10^2$ copies, i.e., increased by one order of magnitude. In addition, the detection velocity for $10^3$ copies was increased by about 4 minutes.

[0147]

As described above, increases in sensitivity and detection velocity were observed as a result of the addition of a ladder-forming oligonucleotide primer to a 1-

step RT-ICAN reaction.

[0148]

Example 2

(1) Examination of effect on detection of polymorphism in human aldehyde dehydrogenase 2 gene (ALDH2) using real-time detection system according to cycling probe method

[0149]

The effect on detection of a polymorphism by real-time detection using the ICAN reaction and the cycling probe method was examined. A gene encoding an enzyme human aldehyde dehydrogenase 2 (GenBank Acc. No.: AH002599) was selected as a subject to be detected. It has been reported that a single nucleotide polymorphism in which glutamic acid at position 487 (GAA) is replaced by lysine (AAA) exists in exon 12 of this gene, and is profoundly related with the individual difference in predisposition concerning alcohol-drinking and involved in the risk of carcinogenesis.

[0150]

A primer ICAN-ALDH2-F (SEQ ID NO:21) which corresponds to a second chimeric oligonucleotide primer and a primer ICAN-ALDH2-R (SEQ ID NO:22) which corresponds to a first chimeric oligonucleotide primer were synthesized using a DNA synthesizer (Applied Biosystems) in order to detect the polymorphism in aldehyde dehydrogenase 2 gene in

an ICAN reaction system. A probe for detecting the normal type ALDH2 wG (SEQ ID NO:23) and a probe for detecting the mutation type ALDH2 mA (SEQ ID NO:24) were synthesized. The probe for detecting the normal type ALDH2 wG is a DNA-RNA-DNA-type oligonucleotide probe having a ROX label (Applied Biosystems) as a fluorescent label attached at the 5' terminus and Eclipse (Epoch Biosciences) as a quenching label attached at the 3' terminus. The probe for detecting the mutant type ALDH2 mA is a DNA-RNA-DNA-type oligonucleotide probe having a FAM label (Applied Biosystems) as a fluorescent label attached at the 5' terminus and Eclipse as a quenching label attached at the 3' terminus.

[0151]

In addition, the following ladder-forming oligonucleotide primers each having a 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -12) of the second chimeric oligonucleotide primer which were designed around the primer ICAN-ALDH2-R were synthesized: ALDH2-TH1 (SEQ ID NO:25; -1 to +15 relative to the chimeric oligonucleotide primer ICAN-ALDH2-R); ALDH2-TH2 (SEQ ID NO:26; -9 to +6 relative to the chimeric oligonucleotide primer ICAN-ALDH2-R); and ALDH2-TH3 (SEQ ID NO:27; -17 to -2 relative to the chimeric oligonucleotide primer ICAN-ALDH2-R).

[0152]

Genomic DNAs as templates were prepared using QIAamp DNA Mini Kit (Qiagen) from blood samples collected after obtaining informed consent for which EDTA was used as an anticoagulant.

[0153]

The reaction conditions for the ICAN reaction were as follows. Briefly, a reaction mixture of a final volume of 25 µl containing the following at final concentrations was prepared: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 5 mM magnesium acetate, 1% dimethyl sulfoxide, 0.04% propylenediamine, 0.11% bovine serum albumin, 600 µM each of dNTPs, 4 U of BcaBEST DNA polymerase, 100 U of Tli RNase HII, 50 pmol each of the primer ICAN-ALDH2-F and the primer ICAN-ALDH2-R, 5.5 pmol of the probe ALDH2 wG, 6 pmol of the probe ALDH2 mA, 100 ng of a genomic DNA as a template and sterile water with or without the addition of 1 pmol of the ladder-forming oligonucleotide primer ALDH2-TH1, ALDH2-TH2 or ALDH2-TH3. The reaction mixture was placed in Smart Cycler (Takara Bio), treated at 70°C for 5 minutes and incubated at 56°C for 60 minutes. The fluorescence intensity was measured during the incubation at 56°C at 1-minute intervals.

[0154]

Furthermore, PCR was conducted using a genomic

DNA as a template and a pair of primers ALDH2-F (SEQ ID NO:28) and ALDH2-R (SEQ ID NO:29). The resulting amplification product was purified using Microcon-30 (Millipore), digested with Eco 57I and subjected to electrophoresis on 3% NuSieve 3:1 agarose gel (Takara Bio) for RFLP typing.

[0155]

The PCR was conducted using TaKaRa ExTaq (Takara Bio). Specifically, a reaction mixture of a final volume of 50 µl containing the following at final concentrations was prepared: 1 x ExTaq buffer, 200 µM each of dNTPs, 1.25 U of ExTaq, 10 pmol each of the primer ALDH2-F and the primer ALDH2-R, 5 µl of a human genomic DNA prepared as a template nucleic acid and sterile water. The reaction mixture was placed in Thermal Cycler SP (Takara Bio) and subjected to heating at 94°C for 30 seconds followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds.

[0156]

Eco 57I is a restriction enzyme that recognizes CTGAAG. It cleaves an amplification product from the normal type of aldehyde dehydrogenase 2, but does not cleave an amplification product from the mutant type.

[0157]

When a genomic DNA sample for which three

fragments (cleaved and uncleaved amplification product) were observed upon PCR RFLP with Eco 57I (i.e., which was considered to be heterozygotic) was used, decrease in Ct value was observed upon addition of ALDH2-TH1, ALDH2-TH2 or ALDH2-TH3 to the reaction system as shown in Tables 1 and 2 below, indicating increase in reactivity. The Ct value is the point at which an amplification curve for a sample intersects a threshold line (100). When ALDH2-TH1 or ALDH2-TH2 was added (not in case of ALDH2-TH3), increase in fluorescence intensity from FAM was observed, indicating increase in detection efficiency.

[0158]

[Table 1]

Table 1: Change in Ct value

| | No addition | ALDH2-TH1 | ALDH2-TH2 | ALDH2-TH3 |
|---|---|---|---|---|
| ROX (wild) | 30.3-33.03 | 21.01-23.87 | 21.06-27.37 | 22.91-24.05 |
| FAM (mutant) | 36.45-40.44 | 22.9-28.0 | 25.65-27.98 | 24.83-34.71 |

[0159]

[Table 2]

Table 2: Fluorescence intensity

| | No addition | ALDH2-TH1 | ALDH2-TH2 | ALDH2-TH3 |
|---|---|---|---|---|
| ROX (wild) | 350-340 | 330-350 | 350-370 | 340-380 |
| FAM (mutant) | 150-245 | 285-370 | 370-380 | 145-250 |

[0160]

When a genomic DNA sample for which two fragments (cleaved amplification product) were observed upon RFLP with Eco 57I (i.e., which was considered to be homozygotic for the normal type) was used, only ROX fluorescence signals from the probe ALDH2 wG were observed. When a

genomic DNA sample for which one fragment (uncleaved amplification product) was observed upon RFLP with Eco 57I (i.e., which was considered to be homozygotic for the mutant type) was used, only 6-FAM fluorescence signals from the probe ALDH2 mA were observed. In these cases, decreases in Ct values were observed, indicating increase in reactivities. The Ct value is the point at which an amplification curve for a sample intersects a threshold line (100).

[0161]

(2) Examination of effect of nucleotide sequence attached at 5' terminus of ladder-forming oligonucleotide primer added to reaction system on detection of polymorphism in ALDH2

[0162]

Regarding the effect on detection of a polymorphism by real-time detection using the ICAN reaction and the cycling probe method as described in Example 2-(1), the effect of a nucleotide sequence attached at the 5' terminus of a ladder-forming oligonucleotide primer to be added to a reaction system upon detection of a gene encoding an enzyme human aldehyde dehydrogenase 2, which was selected as a subject to be detected, was examined.

[0163]

Like in Example 2-(1), the primer ICAN-ALDH2-F,

the primer ICAN-ALDH2-R, the probe for detecting the normal type ALDH2 wG and the probe for detecting the mutation type ALDH2 mA were used for detecting a polymorphism in the aldehyde dehydrogenase 2 gene using an ICAN reaction system. The probe for detecting the normal type ALDH2 wG is a DNA-RNA-DNA-type oligonucleotide probe having a ROX label as a fluorescent label attached at the 5' terminus and Eclipse as a quenching label attached at the 3' terminus. The probe for detecting the mutant type ALDH2 mA is a DNA-RNA-DNA-type oligonucleotide probe having a FAM label as a fluorescent label attached at the 5' terminus and Eclipse as a quenching label attached at the 3' terminus.

[0164]

In addition, a ladder-forming oligonucleotide primer ALDH2-TH2 (-9 to +6 relative to the chimeric primer ICAN-ALDH2-R) having a 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -12) of the primer ICAN-ALDH2-F which was designed around the primer ICAN-ALDH2-R, and a primer ALDH2-TH4 (SEQ ID NO:30) in which the 12-nucleotide sequence attached at the 5' terminus of the primer ALDH2-TH2 is removed were synthesized.

[0165]

Genomic DNAs as templates were prepared using QIAamp DNA Mini Kit (Qiagen) from blood samples collected

after obtaining informed consent for which EDTA was used as an anticoagulant, and one confirmed to be heterozygotic by RFLP typing was used.

[0166]

The reaction conditions for the ICAN reaction were as follows. Briefly, a reaction mixture of a final volume of 25 µl containing the following at final concentrations was prepared: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 5 mM magnesium acetate, 1% dimethyl sulfoxide, 0.04% propylenediamine, 0.11% bovine serum albumin, 600 µM each of dNTPs, 4 U of BcaBEST DNA polymerase, 100 U of Tli RNase HII, 50 pmol each of the primers ICAN-ALDH2-F and ICAN-ALDH2-R, 5.5 pmol of the probe ALDH2 wG, 6 pmol of the probe ALDH2 mA, 100 ng of the genomic DNA as a template and sterile water with or without the addition of 1 pmol of the ladder-forming oligonucleotide primer ALDH2-TH2 or ALDH2-TH4. The reaction mixture was placed in Smart Cycler (Takara Bio), treated at 70°C for 5 minutes and incubated at 56°C for 60 minutes. The fluorescence intensity was measured during the incubation at 56°C at 1-minute intervals.

[0167]

Decrease in Ct value was observed upon addition of ALDH2-TH2 to the reaction system as shown in Tables 3 and 4 below, indicating increase in reactivity. The Ct

value is the point at which an amplification curve for a sample intersects a threshold line (100). Furthermore, increases in fluorescence intensities from ROX and FAM were observed, indicating increase in detection efficiency. On the other hand, change in Ct value or fluorescence intensity was not observed when ALDH2-TH4 was added to the reaction system. That is, when ALDH2-TH4 lacking the 12-nucleotide sequence that anneals to the region upstream (-1 to -12) of the primer ICAN-ALDH2-F, which was attached to the 5' terminus of ALDH-TH2, was added to the reaction system, the increases in reactivity and detection efficiency which were observed for ALDH2-TH2 were not observed.

[0168]

[Table 3]

Table 3: Change in Ct value

| | No addition | ALDH2-TH2 | ALDH2-TH4 |
|---|---|---|---|
| ROX (wild) | 28.2 | 23.9 | 27.2 |
| FAM (mutant) | 34.08 | 24.7 | 34.8 |

[0169]

[Table 4]

Table 4: Fluorescence intensity

| | No addition | ALDH2-TH2 | ALDH2-TH4 |
|---|---|---|---|
| ROX (wild) | 310-320 | 410-420 | 350-360 |
| FAM (mutant) | 270-290 | 465-475 | 230-240 |

[0170]

(3) Examination of effect of nucleotide sequence attached at 5' terminus of ladder-forming oligonucleotide

primer added to reaction system on detection of *Legionella*

[0171]

A ladder-forming oligonucleotide primer having a 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -12 or -13 to -24) of a second chimeric oligonucleotide primer was added, and change in detection velocity of ICAN reaction was examined.

[0172]

The effect on real-time detection using the ICAN reaction and the cycling probe method was examined. A gene encoding *Legionella* Mip (*Legionella pneumophila*, macrophage infectivity potentiator (Mip); GenBank Acc. No.: AF095215) was selected as a subject to be detected.

A chimeric oligonucleotide primer F2 (SEQ ID NO:31) as a second chimeric oligonucleotide primer and a chimeric oligonucleotide primer R2 (SEQ ID NO:32) as a first chimeric oligonucleotide primer were synthesized using a DNA synthesizer (Applied Biosystems) in order to detect the *Legionella* Mip gene using an ICAN reaction system. A probe for detecting the Mip gene Mip4g12 (SEQ ID NO:33) was synthesized. The probe for detecting the Mip gene Mip4g12 is a DNA-RNA-DNA-type oligonucleotide probe having a FAM label as a fluorescent label attached at the 5' terminus and Eclipse as a quenching label attached at the 3' terminus.

[0173]

In addition, the following ladder-forming oligonucleotide primers each having a 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -12 or -13 to -24) of the second chimeric oligonucleotide primer which were designed around the first chimeric oligonucleotide primer were synthesized: R2(-13) (SEQ ID NO:34; -13 to -29 relative to the first chimeric oligonucleotide primer); R2(-13)A12-1 (SEQ ID NO:35; -13 to -29 relative to the first chimeric oligonucleotide primer with a 12-nucleotide sequence of a region upstream (-1 to -12) of the second chimeric oligonucleotide primer attached at the 5' terminus); and R2(-13)A12-2 (SEQ ID NO:36; -13 to -29 relative to the first chimeric oligonucleotide primer with a 12-nucleotide sequence of a region upstream (-13 to -24) of the second chimeric oligonucleotide primer attached at the 5' terminus).

[0174]

*Legionella pneumophila* Control DNA attached to EnviroAmp™ (Perkin Elmer) was used as a genomic DNA as a template.

[0175]

The reaction conditions for the ICAN reaction were as follows. Briefly, a reaction mixture of a final

volume of 25 µl containing the following at final concentrations was prepared: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 5 mM magnesium acetate, 1% dimethyl sulfoxide, 0.11% bovine serum albumin, 500 µM each of dNTPs, 2 U of BcaBEST DNA polymerase, 100 U of Tli RNase HII, 25 pmol each of the chimeric oligonucleotide primer F2 and the chimeric oligonucleotide primer R2, 5 pmol of the probe Mip4g12, 200 copies of Control DNA as a template and sterile water with or without the addition of 1 pmol of R2(-13)A12-1 or R2(-13)A12-2. The reaction mixture was placed in Smart Cycler (Takara Bio), treated at 70°C for 5 minutes and incubated at 53°C for 90 minutes. The fluorescence intensity was measured during the incubation at 53°C at 1-minute intervals.

[0176]

The results are shown in Table 5. Decrease in Ct value was observed upon addition of the ladder-forming oligonucleotide primer R2(-13)A12-1 or R2(-13)A12-2 to the reaction system as shown in Table 5, indicating increase in reactivity. The Ct value is the point at which an amplification curve for a sample intersects a threshold line (100).

[0177]

[Table 5]

Table 5: Change in Ct value

| | No addition | R12(-13)A12-1 | R12(-13)A12-2 |
|---|---|---|---|
| FAM | 74.17-81.12 | 44.11-54.61 | 48.03-55.89 |

[0178]

Example 3

(1) Effect of complementary sequences in regions in nucleic acid as template upstream of respective ICAN primers on ICAN reaction (1)

[0179]

6-nucleotide complementary sequences were inserted into regions in a nucleic acid as a template upstream of 5' termini of respective ICAN primers, and the effect on ICAN reaction was examined. Human c-Ki-ras 2 gene (GenBank Acc. No.: L00045) was selected as a subject to be detected.

[0180]

A template without definite complementary sequences in regions upstream of 5' termini of respective ICAN primers, and a template with 6-nucleotide complementary sequences were prepared as follows.

PCRs were conducted using Human genome (purchased from Clontech) as a template as well as a primer c-Ki-ras/12F (SEQ ID NO:37) and a primer rasT1R (SEQ ID NO:38), or a primer rasT14F (SEQ ID NO:39) and a primer rasT4R (SEQ ID NO:40) which are primers having complementary sequences

attached at the 5' termini of the respective primers. The resulting fragments were blunted, and inserted into a HincII site in a plasmid pUC118 (Takara Bio). Plasmids in which the direction of the primer c-Ki-ras/12F or the primer rasT14F was the same as that of an M13 primer M4 (Takara Bio) which anneals to pUC118 were selected from the resulting plasmids. As a result, a plasmid T7 without definite complementary sequences in regions upstream of the 5' termini of the respective ICAN primers and a plasmid T16 with 6-nucleotide complementary sequences CGCGCG were obtained.

[0181]

The concentration of the prepared plasmid DNA was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$ or $10^3$ copies per µl. 1 µl of one of the template DNAs with varying copy numbers was added to a reaction mixture of 24 µl containing the following at final concentrations: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 30 pmol each of a chimeric oligonucleotide primer c-Ki-ras/12FN3 (SEQ ID NO:41) and a chimeric oligonucleotide primer c-Ki-ras/12RN3 (SEQ ID NO:42), 2.8 U of BcaBEST (Takara Bio) and 2.2 U of Afu RNase HII prepared according to the method as described in Example 7 of WO 02/22831. The reaction mixture was

incubated at 55°C for 60 minutes using Thermal Cycler Personal (Takara Bio). The amplification product was detected by electrophoresis on 3% agarose gel.

[0182]

As a result, when the plasmid T7 without definite complementary sequences in regions upstream of the 5' termini of the respective ICAN primers was used as a template, only a band corresponding to the size of the target region was observed as an amplification product with stable sensitivity of $10^3$ copies. On the other hand, when the plasmid T16 with the sequences CGCGCG which is complementary to upstream regions adjacent to the 5' termini of the respective ICAN primers was used as a template, a band corresponding to the size of the target region as well as high-molecular-weight products in a regular ladder-like pattern were observed with stable sensitivity of 10 copies, indicating increase in sensitivity. The ladder-like product was excised from the gel, subcloned into a HincII site in pUC118, and subjected to nucleotide sequence determination. Then, it was found that, in the ladder-like product, the target regions were connected to each other in the same direction through the complementary sequences CGCGCG in regions upstream of the 5' termini of the respective ICAN primers.

As described above, a reaction of polymerizing

target regions was promoted due to complementary sequences in regions upstream of 5' termini of respective ICAN primers, resulting in increases in sensitivity.

[0183]

(2) Effect of complementary sequences in regions in nucleic acid as template upstream of respective ICAN primers on ICAN reaction (2)

[0184]

The effect of 3-nucleotide complementary sequences in regions in a nucleic acid as a template upstream of 5' termini of respective ICAN primers on ICAN reaction was examined. Gonococcus cppB (*Neisseria gonorrhoeae* cryptic plasmid protein (cppB); GenBank Acc. No.: M10316) gene was selected as a subject to be detected.

[0185]

A template was prepared as follows.

PCR was conducted using gonococcus genome (purchased from Summit Pharmaceuticals International Corporation) as a template as well as a primer PJDBF (SEQ ID NO:43) and a primer PJDBR (SEQ ID NO:44). The resulting fragment was blunted, and inserted into a HincII site in a plasmid pUC118 (Takara Bio). A plasmid in which the direction of the primer PJDBF was the same as that of an M13 primer M4 (Takara Bio) which anneals to pUC118 was selected from the resulting plasmids. As a result, a

plasmid Cp13 was obtained.

A primer PJDB0FN3 (SEQ ID NO:45) and a primer PJDB0RN3 (SEQ ID NO:46) were designed in order to amplify the inserted region using the plasmid Cp13 as a template. In this case, the strand of the target region for the primer PJDB0FN3 did not contain the sequence GTC which is a sequence complementary to the upstream 3-nucleotide sequences GAC adjacent to the 5' terminus of the primer PJDB0RN3. The sequence existed at a position 34 nucleotides upstream of the 5' terminus of the primer PJDB0FN3. The effect of the 3-nucleotide sequence on ICAN was examined.

[0186]

The concentration of the prepared plasmid DNA prepared as described above was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$ or $10^8$ copies per µl. 1 µl of one of the template DNAs with varying copy numbers was added to a reaction mixture of 24 µl containing the following at final concentrations: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 30 pmol each of the chimeric oligonucleotide primer PJDB0FN3 and the chimeric oligonucleotide primer PJDB0RN3, 2.8 U of BcaBEST (Takara Bio) and 2.2 U of Afu RNase HII. The reaction

mixture was incubated at 55°C for 60 minutes using Thermal Cycler Personal (Takara Bio). The amplification product was detected by electrophoresis on 3% agarose gel.

[0187]

As a result, a band corresponding to the size of the target region as well as high-molecular-weight products in a regular ladder-like pattern were observed with high sensitivity ($10^2$ copies). The ladder-like product was excised from the gel, subcloned into a HincII site in pUC118, and subjected to nucleotide sequence determination. Then, it was found that, in the ladder-like product, the target regions were connected to each other in the same direction in a regular pattern through the 3-nucleotide complementary sequences GAC and GTC in regions upstream of the 5' termini of the respective ICAN primers. The ladder-like amplification product contained the target region between the ICAN primers and the 34-nucleotide region upstream of the 5' terminus of the chimeric oligonucleotide primer PJDB0FN3 between the target regions in a regular pattern.

[0188]

As described above, it was confirmed that a reaction of polymerizing target regions was promoted due to 3-nucleotide complementary sequences in regions upstream of 5' termini of respective ICAN primers, enabling highly

sensitive amplification. In addition, it was shown that if an amplification product is to be detected using hybridization with a probe, probe positions can be selected from a region between respective ICAN primers or a region between an ICAN primer and a complementary sequence in a region upstream of the 5' terminus of an ICAN primer.

[0189]

Example 4

Sensitivity and detection velocity of RT-ICAN were examined using a ladder-forming oligonucleotide primer having a 6-, 9- or 12-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -6, -1 to -9 or -1 to -12) of a second chimeric oligonucleotide primer as a reverse transcription primer.

[0190]

First, the concentration of the template RNA prepared in Example 1-(1) was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ copies per µl.

A reaction mixture of 10 µl containing the following at final concentrations was prepared: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 1 pmol of a ladder-forming oligonucleotide primer 215R, a primer A6-215R (SEQ ID

NO:47), a primer A9-215R (SEQ ID NO:48) or a primer A12-215R as a reverse transcription primer, 50 U of RTase M-MLV (Takara Bio) and 1 µl of one of the template RNAs with varying copy numbers.

[0191]

The reaction mixture was placed in Thermal Cycler Personal (Takara Bio), incubated at 45°C for 10 minutes, and then cooled to 4°C. After reverse transcription reaction, 15 µl of a reaction mixture containing the following was added to 10 µ of the reaction mixture: mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 25 pmol each of a primer B134FN3(16) as a second chimeric oligonucleotide primer and a primer 205RN3(16) as a first chimeric oligonucleotide primer, 11 U of BcaBEST (Takara Bio), 0.05 U of Tli RNase HII and SYBR Green. An ICAN reaction was carried out at 55°C using Rotor gene (Corbett Research) to detect the amplification product in a real-time manner. The reaction product was also detected by electrophoresis on 3% agarose gel.

[0192]

When the ladder-forming oligonucleotide primer A6-215R having a 6-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -6) of the second chimeric oligonucleotide primer was used, the

sensitivity was $10^2$ copies. The attachment of the 6-nucleotide sequence increased the detection velocity by 0.9 minute.

When the ladder-forming oligonucleotide primer A9-215R having a 9-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -9) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^2$ copies. The attachment of the 9-nucleotide sequence increased the detection velocity by 1.6 minute.

When the ladder-forming oligonucleotide primer A12-215R having a 12-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -12) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^1$ copies. The attachment of the 12-nucleotide sequence increased the detection velocity by 3.7 minute.

[0193]

As described above, it was confirmed that a sequence of more nucleotides attached at the 5' terminus of a ladder-forming oligonucleotide primer that anneals to a region upstream of a second chimeric oligonucleotide primer results in a more effect.

[0194]

Example 5

Sensitivity of RT-ICAN was examined using a ladder-forming oligonucleotide primer having a 12- or 18-nucleotide sequence attached at the 5' terminus that anneals to a region upstream (-1 to -12 or -1 to -18) of a second chimeric oligonucleotide primer as a reverse transcription primer.

[0195]

First, the concentration of the template RNA prepared in Example 1-(1) was calculated on the basis of the OD260 value and adjusted to $10^0$, $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ copies per µl.

A reaction mixture of 10 µl containing the following at final concentrations was prepared: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 1 pmol of a ladder-forming oligonucleotide primer 205RN3(18), a primer A12-205R or a primer A18-205R (SEQ ID NO:49) as a reverse transcription primer, 50 U of RTase M-MLV (Takara Bio) and 1 µl of one of the template RNAs with varying copy numbers.

[0196]

The reaction mixture was placed in Thermal Cycler Personal (Takara Bio), incubated at 45°C for 10 minutes, and then cooled to 4°C. After reverse transcription reaction, 15 µl of a reaction mixture containing the

following at final concentrations was added to 10 µl of the reaction mixture: 32 mM HEPES-KOH buffer (pH 7.8), 100 mM potassium acetate, 1% dimethyl sulfoxide, 0.11% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 25 pmol each of a primer B134FN3(18) as a second chimeric oligonucleotide primer and a primer 205RN3(18) as a first chimeric oligonucleotide primer, 11 U of BcaBEST (Takara Bio) and 0.05 U of Tli RNase HII. An ICAN reaction was carried out at 55°C using Thermal Cycler Personal (Takara Bio). The amplification products were detected by electrophoresis on 3% agarose gel.

[0197]

As a result, the sensitivity of RT-ICAN with reverse transcription using the primer 205RN3 was $10^2$ copies. When the ladder-forming oligonucleotide primer A12-205R for the same position having a 12-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -12) of the second chimeric oligonucleotide primer or the ladder-forming oligonucleotide primer A18-205R for the same position having a 18-nucleotide sequence attached at the 5' terminus that anneals to the region upstream (-1 to -18) of the second chimeric oligonucleotide primer was used, the sensitivity was $10^1$ copies, i.e., increased by one order of magnitude. In addition, it was confirmed that

amplification products in a regular ladder-like pattern resulting from connection through annealing sequences in regions upstream of the primers were obtained by amplification using A12-205R or A18-205R.

[0198]

As described above, even if the number of nucleotides annealing to a region upstream of a second chimeric oligonucleotide primer was changed, ladder-like amplification through the region occurred, resulting in increased sensitivity.

Industrial Applicability

[0199]

The present invention provides an amplification method superior to conventional isothermal nucleic acid amplification methods as well as a composition and a kit containing a primer used for the method.

Sequence Listing Free Text

[0200]

SEQ ID NO. 1: A portion of SARS coronavirus genomic RNA reverse transcripted to DNA. "nucleotide 1 to 5 is HindIII restriction site- nucleotide 238 to 242 is BamHI restriction site."

SEQ ID NO. 2: Designed chimeric oligonucleotide

primer designated as 205RN3(18) for synthesizing cDNA from mRNA, and to amplify a portion of SARS coronavirus genome. "nucleotides 16 to 18 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 3: Designed oligonucleotide primer designated as A12-205R for synthesizing cDNA from mRNA.

SEQ ID NO. 4: Designed oligonucleotide primer designated as 215R for synthesizing cDNA from mRNA.

SEQ ID NO. 5: Designed oligonucleotide primer designated as A12-215R for synthesizing cDNA from mRNA, and to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 6: Designed oligonucleotide primer designated as A12-223R for synthesizing cDNA from mRNA, and to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 7: Designed chimeric oligonucleotide primer designated as 134FN3(18) to amplify a portion of SARS coronavirus genome. "nucleotides 16 to 18 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 8: Designed oligonucleotide primer designated as A12(-10)-215R for synthesizing cDNA from mRNA, and to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 9: Designed oligonucleotide primer designated as A12(-20)-215R for synthesizing cDNA from mRNA, and to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 10: Designed oligonucleotide primer designated as A12(6)-215R for synthesizing cDNA from mRNA, and to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 11: Designed oligonucleotide primer designated as A12(12)-215R for synthesizing cDNA from mRNA, and to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 12: Designed chimeric oligonucleotide primer designated as B134FN3(16) to amplify a portion of SARS coronavirus genome. "nucleotides 14 to 16 are ribonucleotides- other nucleotides are deoxyribonucleotides." "5'-end is labeled with biotin."

SEQ ID NO. 13: Designed chimeric oligonucleotide primer designated as 205RN3(16) to amplify a portion of SARS coronavirus genome. "nucleotides 14 to 16 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 14: Designed oligonucleotide primer designated as A6(-10)-215R for synthesizing cDNA from mRNA, and to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 15: Designed oligonucleotide primer designated as A9(-10)-215R for synthesizing cDNA from mRNA, and to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 16: Designed oligonucleotide probe designated as SARS-BNI-B for detecting an amplified a portion of SARS coronavirus genome. "5'-end is labeled

with FITC."

SEQ ID NO. 17: Designed chimeric oligonucleotide primer designated as 160FN3 to amplify a portion of SARS coronavirus genome. "nucleotides 16 to 18 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 18: Designed chimeric oligonucleotide primer designated as 241RN3 to amplify a portion of SARS coronavirus genome. "nucleotides 12 to 14 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 19: Designed chimeric oligonucleotide primer designated as (A12)241RN3 to amplify a portion of SARS coronavirus genome. "nucleotides 18 to 21 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 20: Designed chimeric oligonucleotide primer designated as 134FN3(16) to amplify a portion of SARS coronavirus genome. "nucleotides 14 to 16 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 21: Designed chimeric oligonucleotide primer designated as ICAN-ALDH2-F to amplify a portion of human aldehyde dehydrogenase 2 gene. "nucleotides 18 to 20 are ribonucleotides- other nucleotides are

deoxyribonucleotides."

SEQ ID NO. 22: Designed chimeric oligonucleotide primer designated as ICAN-ALDH2-R to amplify a portion of human aldehyde dehydrogenase 2 gene. "nucleotides 18 to 20 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 23: Designed chimeric oligonucleotide probe designated as ALDH2 wG probe for detecting an amplified a portion of native human aldehyde dehydrogenase 2 gene. "nucleotides 11 is ribonucleotide- other nucleotides are deoxyribonucleotides." "5'-end is labeled with ROX, and 3'-end is labeled with Eclipse."

SEQ ID NO. 24: Designed chimeric oligonucleotide probe designated as ALDH2 mA probe for detecting an amplified a portion of mutant human aldehyde dehydrogenase 2 gene. "nucleotides 11 is ribonucleotide- other nucleotides are deoxyribonucleotides." "5'-end is labeled with FAM, and 3'-end is labeled with Eclipse."

SEQ ID NO. 25: Designed oligonucleotide primer designated as ALDH2-TH1 to amplify a portion of human aldehyde dehydrogenase 2 gene.

SEQ ID NO. 26: Designed oligonucleotide primer designated as ALDH2-TH2 to amplify a portion of human aldehyde dehydrogenase 2 gene.

SEQ ID NO. 27: Designed oligonucleotide primer

designated as ALDH2-TH3 to amplify a portion of human aldehyde dehydrogenase 2 gene.

SEQ ID NO. 28: Designed oligonucleotide PCR primer designated as ALDH2-F to amplify a portion of human aldehyde dehydrogenase 2 gene.

SEQ ID NO. 29: Designed oligonucleotide PCR primer designated as ALDH2-R to amplify a portion of human aldehyde dehydrogenase 2 gene.

SEQ ID NO. 30: Designed oligonucleotide primer designated as ALDH2-TH4 to amplify a portion of human aldehyde dehydrogenase 2 gene.

SEQ ID NO. 31: Designed chimeric oligonucleotide primer designated as F2 to amplify a portion of Legionella pneumophila mip gene. "nucleotides 15 to 17 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 32: Designed chimeric oligonucleotide primer designated as R2 to amplify a portion of Legionella pneumophila mip gene. "nucleotides 15 to 17 are ribonucleotides- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 33: Designed chimeric oligonucleotide probe designated as Mip4g12 probe for detecting an amplified a portion of Legionella pneumophila mip gene. "nucleotides 4 is ribonucleotide- other nucleotides are

deoxyribonucleotides." "5'-end is labeled with FAM, and 3'-end is labeled with Eclipse."

SEQ ID NO. 34: Designed oligonucleotide primer designated as R2(-13) to amplify a portion of Legionella pneumophila mip gene.

SEQ ID NO. 35: Designed oligonucleotide primer designated as R2(-13)A12-1 to amplify a portion of Legionella pneumophila mip gene.

SEQ ID NO. 36: Designed oligonucleotide primer designated as R2(-13)A12-2 to amplify a portion of Legionella pneumophila mip gene.

SEQ ID NO. 37: Designed oligonucleotide PCR primer designated as c-Ki-ras/12F to amplify a portion of human c-Ki-ras2 gene.

SEQ ID NO. 38: Designed oligonucleotide PCR primer designated as rasT1R to amplify a portion of human c-Ki-ras2 gene.

SEQ ID NO. 39: Designed oligonucleotide PCR primer designated as rasT14F to amplify a portion of human c-Ki-ras2 gene.

SEQ ID NO. 40: Designed oligonucleotide PCR primer designated as rasT4R to amplify a portion of human c-Ki-ras2 gene.

SEQ ID NO. 41: Designed chimeric oligonucleotide primer designated as c-Ki-ras/12FN3 to amplify a portion of

human c-Ki-ras2 gene. "nucleotides 18 to 20 are ribonucleotide- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 42: Designed chimeric oligonucleotide primer designated as c-Ki-ras/12RN3 to amplify a portion of human human c-Ki-ras2 gene. "nucleotides 18 to 20 are ribonucleotide- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 43: Designed oligonucleotide primer designated as PJDBF to amplify a portion of Neisseria gonorrhoeae cppB gene.

SEQ ID NO. 44: Designed oligonucleotide primer designated as PJDBR to amplify a portion of Neisseria gonorrhoeae cppB gene.

SEQ ID NO. 45: Designed chimeric oligonucleotide primer designated as PJDB0FN3 to amplify a portion of Neisseria gonorrhoeae cppB gene. "nucleotides 18 to 20 are ribonucleotide- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 46: Designed chimeric oligonucleotide primer designated as PJDB0RN3 to amplify a portion of Neisseria gonorrhoeae cppB gene. "nucleotides 15 to 17 are ribonucleotide- other nucleotides are deoxyribonucleotides."

SEQ ID NO. 47: Designed oligonucleotide primer

designated as A6-215R to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 48: Designed oligonucleotide primer designated as A9-215R to amplify a portion of SARS coronavirus genome.

SEQ ID NO. 49: Designed oligonucleotide primer designated as A18-205R to amplify a portion of SARS coronavirus genome.